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SUBTILASE VARIANTS HAVING A REDUCED TENDENCY TOWARDS INHIBITION BY SUBSTANCES PRESENT IN EGGS

TECHNICAL FIELD

The present invention relates to novel subtilase variants having a reduced tendency towards inhibition by substances present in eggs, such as trypsin inhibitor type IV-0. In particular, the present invention relates to novel subtilase variants where the variants comprise at least one additional amino acid residue between positions 42-43, 51-55, 155-160, 187-189, 217-218 or 218-219 (in BASBPN numbering, *vide infra*). These subtilase variants are useful exhibiting excellent or improved wash performance on egg stains when used in e.g. cleaning or detergent compositions, such as laundry detergent compositions and dishwash composition, including automatic dishwash compositions. The present invention also relates to isolated DNA sequences encoding the variants, expression vectors, host cells, and methods for producing and using the variants of the invention. Further, the present invention relates to cleaning and detergent compositions comprising the variants of the invention.

BACKGROUND OF THE INVENTION

In the detergent industry enzymes have for more than 30 years been implemented in washing formulations. Enzymes used in such formulations comprise proteases, lipases, amylases, cellulases, as well as other enzymes, or mixtures thereof. Commercially most important enzymes are proteases.

An increasing number of commercially used proteases are protein engineered variants of naturally occurring wild type proteases, e.g. DURAZYM® (Novo Nordisk A/S), RELASE® (Novo Nordisk A/S), MAXAPEM® (Gist-Brocades N.V.), PURAFECT® (Genencor International, Inc.).

Further, a number of protease variants are described in the art. A thorough list of prior art protease variants is given in WO 99/27082.

However, even though a number of useful protease variants have been described, there is still a need for new improved proteases or protease variants for a number of industrial uses.

In particular, the problem of removing egg stains from e.g. laundry or hard surfaces has been pronounced due to the fact that many proteases are inhibited by substances present in the egg white. Examples of such substances include trypsin inhibitor type IV-0 (Ovo-inhibitor) and trypsin inhibitor type III-0 (Ovomucoid).

Therefore, an object of the present invention, is to provide improved subtilase variants, which are not, or which are only to a limited extent, inhibited by such substances. A further object of the present invention is to provide improved subtilase variants, which are suitable for removal of egg stains from, for example, laundry and/or hard surfaces.

SUMMARY OF THE INVENTION

Thus, in a first aspect the present invention relates to a subtilase variant selected from the group consisting of

a subtilase variant comprising an insertion of at least one additional amino acid residue between positions 42 and 43 (BASBPN numbering);

a subtilase variant comprising an insertion of at least one additional amino acid residue between positions 51 and 55 (BASBPN numbering);

a subtilase variant comprising an insertion of at least one additional amino acid residue between positions 155 and 160 (BASBPN numbering);

a subtilase variant comprising an insertion of at least one additional amino acid residue between positions 187 and 189 (BASBPN numbering);

a subtilase variant comprising an insertion of at least one additional amino acid residue between positions 216 and 217 (BASBPN numbering); and

a subtilase variant comprising an insertion of at least one additional amino acid residue between positions 217 and 218 (BASBPN numbering); and

a subtilase variant comprising an insertion of at least one additional amino acid residue between positions 218 and 219 (BASBPN numbering).

In a second aspect the present invention relates to an isolated DNA sequence encoding a subtilase variant of the invention.

In a third aspect the present invention relates to an expression vector comprising the isolated DNA sequence of the invention.

In a fourth aspect the present invention relates to a microbial host cell transformed with the expression vector of the invention.

In a fifth aspect the present invention relates to a method for producing a subtilase variant according to the invention, wherein a host according to the invention is cultured under conditions conducive to the expression and secretion of the variant, and the variant is recovered.

In an sixth aspect the present invention relates to a cleaning or detergent composition, preferably a laundry or dishwash composition, comprising the variant of the invention.

In a seventh aspect the present invention relates to a method for removal of egg stains from a hard surface or from laundry, the method comprising contacting the egg stain-containing hard surface or the egg stain-containing laundry with a cleaning or detergent composition, preferably a laundry or dishwash composition, which contains a subtilase variant according to the invention.

In an eight aspect the present invention relates to the use of a cleaning or detergent composition, preferably a laundry or dishwash composition, containing a subtilase variant according to the invention for removal of egg stains from laundry or from hard surfaces.

Concerning alignment and numbering reference is made to Fig. 1 which shows an alignments between subtilisin BPN'(a) (BASBPN) and subtilisin 309 (BLSAVI)(b).

These alignments are in this patent application used as a reference for numbering the residues.

DEFINITONS

Prior to discussing this invention in further detail, the following terms and conventions will first be defined.

NOMENCLATURE OF AMINO ACIDS

A	=	Ala	=	Alanine
V	=	Val	=	Valine
L	=	Leu	=	Leucine

I	=	Ile	=	Isoleucine
P	=	Pro	=	Proline
F	=	Phe	=	Phenylalanine
W	=	Trp	=	Tryptophan
M	=	Met	=	Methionine
G	=	Gly	=	Glycine
S	=	Ser	=	Serine
T	=	Thr	=	Threonine
C	=	Cys	=	Cysteine
Y	=	Tyr	=	Tyrosine
N	=	Asn	=	Asparagine
Q	=	Gln	=	Glutamine
D	=	Asp	=	Aspartic Acid
E	=	Glu	=	Glutamic Acid
K	=	Lys	=	Lysine
R	=	Arg	=	Arginine
H	=	His	=	Histidine
X	=	Xaa	=	Any amino acid

NOMENCLATURE OF NUCLEIC ACIDS

A	=	Adenine
G	=	Guanine
C	=	Cytosine
T	=	Thymine (only in DNA)
U	=	Uracil (only in RNA)

NOMENCLATURE AND CONVENTIONS FOR DESIGNATION OF VARIANTS

In describing the various subtilase enzyme variants produced or contemplated according to the invention, the following nomenclatures and conventions have been adapted for ease of reference:

A frame of reference is first defined by aligning the isolated or parent enzyme with subtilisin BPN' (BASBPN).

The alignment can be obtained by the GAP routine of the GCG package version 9.1 to number the variants using the following parameters: gap creation penalty = 8 and gap extension penalty = 8 and all other parameters kept at their default values.

Another method is to use known recognized alignments between subtilases, such as the alignment indicated in WO 91/00345. In most cases the differences will not be of any importance.

Thereby a number of deletions and insertions will be defined in relation to BASBPN. In Fig. 1, subtilisin 309 has 6 deletions in positions 36, 58, 158, 162, 163, and 164 in comparison to BASBPN. These deletions are in Fig. 1 indicated by asterixes (*).

The various modifications performed in a parent enzyme is indicated in general using three elements as follows:

Original amino acid position substituted amino acid

The notation G195E thus means a substitution of a glycine in position 195 with a glutamic acid.

In the case where the original amino acid residue may be any amino acid residue, a short hand notation may at times be used indicating only the position and substituted amino acid:

Position substituted amino acid

Such a notation is particular relevant in connection with modification(s) in homologous subtilases (*vide infra*).

Similarly when the identity of the substituting amino acid residue(s) is immaterial:

Original amino acid position

When both the original amino acid(s) and substituted amino acid(s) may comprise any amino acid, then only the position is indicated, *e.g.*: 170.

When the original amino acid(s) and/or substituted amino acid(s) may comprise more than one, but not all amino acid(s), then the selected amino acids are indicated inside brackets:

Original amino acid position {substituted amino $acid_1$, . . . , substituted amino $acid_n$}

For specific variants the specific three or one letter codes are used, including the codes Xaa and X to indicate any amino acid residue.

SUBSTITUTIONS:

The substitution of glutamic acid for glycine in position 195 is designated as:

Gly195Glu or G195E

or the substitution of any amino acid residue acid for glycine in position 195 is designated as:

Gly195Xaa or G195X

or

Gly195 or G195

The substitution of serine for any amino acid residue in position 170 would thus be designated

Xaa170Ser or X170S.

or

170Ser or 170S

Such a notation is particular relevant in connection with modification(s) in homologous subtilases (*vide infra*). 170Ser is thus meant to comprise *e.g.* both a Lys170Ser modification in BASBPN and Arg170Ser modification in BLSAVI (cf. Fig. 1).

For a modification where the original amino acid(s) and/or substituted amino acid(s) may comprise more than one, but not all amino acid(s), the substitution of glycine, alanine, serine or threonine for arginine in position 170 would be indicated by

Arg170{Gly,Ala,Ser,Thr} or R170{G,A,S,T}

to indicate the variants

R170G, R170A, R170S, and R170T.

DELETIONS:

A deletion of glycine in position 195 will be indicated by:

Gly195* or G195*

Correspondingly the deletion of more than one amino acid residue, such as the deletion of glycine and leucine in positions 195 and 196 will be designated

Gly195*+Leu196* or G195*+L196*

INSERTIONS:

The insertion of an additional amino acid residue such as *e.g.* a lysine after G195 is indicated by:

Gly195GlyLys or G195GK;

or, when more than one amino acid residue is inserted, such as *e.g.* a Lys, Ala and Ser after G195 this will be indicated as:

Gly195GlyLysAlaSer or G195GKAS

In such cases the inserted amino acid residue(s) are numbered by the addition of lower case letters to the position number of the amino acid residue preceding the inserted amino acid residue(s). In the above example the sequences 194 to 196 would thus be:

	194	195	196			
BLSAVI	A	G	L			
	194	195	195a	195b	195c	196
Variant	A	G	K	A	S	L

In cases where an amino acid residue identical to the existing amino acid residue is inserted it is clear that a degeneracy in the nomenclature arises. If for example a glycine is inserted after the glycine in the above example this would be indicated by G195GG. The same actual change could just as well be indicated as A194AG for the change from

	194	195	196
BLSAVI	A	G	L

to

	194	195	195a	196
Variant	A	- G -	G	- L
	194	194a	195	196

Such instances will be apparent to the skilled person, and the indication G195GG and corresponding indications for this type of insertions are thus meant to comprise such equivalent degenerate indications.

FILLING A GAP:
Where a deletion in an enzyme exists in the reference comparison with the subtilisin BPN' sequence used for the numbering, an insertion in such a position is indicated as:

*36Asp or *36D

for the insertion of an aspartic acid in position 36

MULTIPLE MODIFICATIONS:
Variants comprising multiple modifications are separated by pluses, *e.g.*:

Arg170Tyr+Gly195Glu or R170Y+G195E

representing modifications in positions 170 and 195 substituting tyrosine and glutamic acid for arginine and glycine, respectively.

Thus, Tyr167{Gly,Ala,Ser,Thr}+Arg170{Gly,Ala,Ser,Thr} designates the following variants:

Tyr167Gly+Arg170Gly, Tyr167Gly+Arg170Ala,
Tyr167Gly+Arg170Ser, Tyr167Gly+Arg170Thr,

Tyr167Ala+Arg170Gly, Tyr167Ala+Arg170Ala,
Tyr167Ala+Arg170Ser, Tyr167Ala+Arg170Thr,
Tyr167Ser+Arg170Gly, Tyr167Ser+Arg170Ala,
Tyr167Ser+Arg170Ser, Tyr167Ser+Arg170Thr,
Tyr167Thr+Arg170Gly, Tyr167Thr+Arg170Ala,
Tyr167Thr+Arg170Ser, and Tyr167Thr+Arg170Thr.

This nomenclature is particular relevant relating to modifications aimed at substituting, replacing, inserting or deleting amino acid residues having specific common properties, such as residues of positive charge (K, R, H), negative charge (D, E), or conservative amino acid modification(s) of *e.g.* Tyr167{Gly,Ala,Ser,Thr}+Arg170{Gly,Ala,Ser,Thr}, which signifies substituting a small amino acid for another small amino acid. See section "Detailed description of the invention" for further details.

Proteases

Enzymes cleaving the amide linkages in protein substrates are classified as proteases, or (interchangeably) peptidases (see Walsh, 1979, *Enzymatic Reaction Mechanisms*. W.H. Freeman and Company, San Francisco, Chapter 3).

Numbering of amino acid positions/residues

If nothing else is mentioned the amino acid numbering used herein correspond to that of the subtilase BPN' (BASBPN) sequence. For further description of the BPN' sequence, see Fig. 1 or Siezen *et al.*, *Protein Engng.* **4** (1991) 719-737.

Serine proteases

A serine protease is an enzyme which catalyzes the hydrolysis of peptide bonds, and in which there is an essential serine residue at the active site (White, Handler and Smith, 1973

"*Principles of Biochemistry*," Fifth Edition, McGraw-Hill Book Company, NY, pp. 271-272).

The bacterial serine proteases have molecular weights in the 20,000 to 45,000 Dalton range. They are inhibited by diisopropylfluorophosphate. They hydrolyze simple terminal esters and are similar in activity to eukaryotic chymotrypsin, also a serine protease. A more narrow term, alkaline protease, covering a sub-group, reflects the high pH optimum of some of the serine proteases, from pH 9.0 to 11.0 (for review, see Priest (1977) *Bacteriological Rev.* **41** 711-753).

Subtilases

A sub-group of the serine proteases tentatively designated subtilases has been proposed by Siezen *et al.*, *Protein Engng.* **4** (1991) 719-737 and Siezen et al. *Protein Science* **6** (1997) 501-523. They are defined by homology analysis of more than 170 amino acid sequences of serine proteases previously referred to as subtilisin-like proteases. A subtilisin was previously often defined as a serine protease produced by Gram-positive bacteria or fungi, and according to Siezen *et al.* now is a subgroup of the subtilases. A wide variety of subtilases have been identified, and the amino acid sequence of a number of subtilases has been determined. For a more detailed description of such subtilases and their amino acid sequences reference is made to Siezen *et al.*(1997).

One subgroup of the subtilases, I-S1 or "true" subtilisins, comprises the "classical" subtilisins, such as subtilisin 168 (BSS168), subtilisin BPN', subtilisin Carlsberg (ALCALASE®, NOVO NORDISK A/S), and subtilisin DY (BSSDY).

A further subgroup of the subtilases, I-S2 or high alkaline subtilisins, is recognized by Siezen *et al.* (*supra*). Sub-group

I-S2 proteases are described as highly alkaline subtilisins and comprises enzymes such as subtilisin PB92 (BAALKP) (MAXACAL®, Gist-Brocades NV), subtilisin 309 (SAVINASE®, NOVO NORDISK A/S), subtilisin 147 (BLS147) (ESPERASE®, NOVO NORDISK A/S), and alkaline elastase YaB (BSEYAB).

"SAVINASE®"

SAVINASE® is marketed by NOVO NORDISK A/S. It is subtilisin 309 from *B. Lentus* and differs from BAALKP only in one position (N87S, see Fig. 1 herein). SAVINASE® has the amino acid sequence designated b) in Fig. 1.

Parent subtilase

The term "parent subtilase" describes a subtilase defined according to Siezen et al. (1991 and 1997). For further details see description of "SUBTILASES" immediately above. A parent subtilase may also be a subtilase isolated from a natural source, wherein subsequent modifications have been made while retaining the characteristic of a subtilase. Furthermore, a parent subtilase may also be a subtilase which has been prepared by the DNA shuffling technique, such as described by J.E. Ness *et al.*, Nature Biotechnology, **17**, 893-896 (1999). Alternatively the term "parent subtilase" may be termed "wild type subtilase".

Modification(s) of a subtilase variant

The term "modification(s)" used herein is defined to include chemical modification of a subtilase as well as genetic manipulation of the DNA encoding a subtilase. The modification(s) can be replacement(s) of the amino acid side chain(s), substitution(s), deletion(s) and/or insertions in or at the amino acid(s) of interest.

Subtilase variant

In the context of this invention, the term subtilase variant or mutated subtilase means a subtilase that has been produced by an organism which is expressing a mutant gene derived from a parent microorganism which possessed an original or parent gene and which produced a corresponding parent enzyme, the parent gene having been mutated in order to produce the mutant gene from which said mutated subtilase protease is produced when expressed in a suitable host.

Homologous subtilase sequences

The homology between two amino acid sequences is in this context described by the parameter "identity".

In order to determine the degree of identity between two subtilases the GAP routine of the GCG package version 9.1 can be applied (*infra*) using the same settings. The output from the routine is besides the amino acid alignment the calculation of the "Percent Identity" between the two sequences.

Based on this description it is routine for a person skilled in the art to identify suitable homologous subtilases and corresponding homologous active site loop regions, which can be modified according to the invention.

Isolated DNA sequence

The term "isolated", when applied to a DNA sequence molecule, denotes that the DNA sequence has been removed from its natural genetic milieu and is thus free of other extraneous or unwanted coding sequences, and is in a form suitable for use within genetically engineered protein production systems. Such isolated molecules are those that are separated from their natural environment and include cDNA and genomic clones. Isolated DNA molecules of the present invention are free of

other genes with which they are ordinarily associated, but may include naturally occurring 5' and 3' untranslated regions such as promoters and terminators. The identification of associated regions will be evident to one of ordinary skill in the art (see for example, Dynan and Tijan, Nature 316:774-78, 1985). The term "an isolated DNA sequence" may alternatively be termed "a cloned DNA sequence".

Isolated protein

When applied to a protein, the term "isolated" indicates that the protein has been removed from its native environment.

In a preferred form, the isolated protein is substantially free of other proteins, particularly other homologous proteins (*i.e.* "homologous impurities" (see below)).

An isolated protein is more than 10% pure, preferably more than 20% pure, more preferably more than 30% pure, as determined by SDS-PAGE. Further it is preferred to provide the protein in a highly purified form, i.e., more than 40% pure, more than 60% pure, more than 80% pure, more preferably more than 95% pure, and most preferably more than 99% pure, as determined by SDS-PAGE.

The term "isolated protein" may alternatively be termed "purified protein".

Homologous impurities

The term "homologous impurities" means any impurity (e.g. another polypeptide than the subtilase of the invention), which originate from the homologous cell where the subtilase of the invention is originally obtained from.

Obtained from

The term "obtained from" as used herein in connection with a specific microbial source, means that the polynucleotide and/or subtilase produced by the specific source, or by a cell in which a gene from the source has been inserted.

Substrate

The term "substrate" used in connection with a substrate for a protease should be interpreted in its broadest form as comprising a compound containing at least one peptide (amide) bond susceptible to hydrolysis by a subtilisin protease.

Product

The term "product" used in connection with a product derived from a protease enzymatic reaction should, in the context of the present invention, be interpreted to include the products of a hydrolysis reaction involving a subtilase protease. A product may be the substrate in a subsequent hydrolysis reaction.

Wash Performance

In the present context the term "wash performance" is used as an enzyme's ability to remove egg stains present on the object to be cleaned during e.g. wash or hard surface cleaning. See also the "Model Detergent Wash Performance Test" in Example 3 herein.

Performance Factor

The term "Performance Factor" is defined with respect to the below formula

$$P = R_{variant} - R_{parent}$$

wherein P is the Performance Factor, $R_{variant}$ is the reflectance (measured at 460 nm) of the test material after being treated

with a subtilase variant as described in the "Model Detergent Wash Performance Test", and R_{parent} is the reflectance (measured at 460 nm) of the test material after being treated with the corresponding parent subtilase as described in the "Model Detergent Wash Performance Test". For further details, see the "Model Detergent Wash Performance Test" in Example 3 herein.

Residual Activity

The term "Residual Activity" is defined as described in the "Ovo-inhibition Assay" herein (see Example 3).

BRIEF DESCRIPTION OF THE DRAWING

Fig. 1 shows an alignment between subtilisin BPN' (a) and Savinase®(b) using the GAP routine mentioned above.

DETAILED DESCRIPTION OF THE INVENTION

The present inventors have found that subtilisin variants, wherein certain regions are longer than those presently known, are inhibited to a significant smaller extent than the parent subtilase and, consequently, the variants according to the invention exhibit improved wash performance with respect to removal of egg stains.

The identification thereof was done in constructing subtilisin variants, especially of the subtilisin 309 (BLSAVI or Savinase®). Without being limited to any specific theory it is presently believed that due to steric hindrance and/or conformational changes, binding of the egg white inhibitor in the substrate binding region of the subtilase variant is impeded.

Thus, variants which are contemplated as being suitable for the uses described herein are such variants where, when compared to the wild-type subtilase, one or more amino acid residues has

been inserted in one or more of the following positions: between positions 42 and 43, between positions 51 and 52, between positions 52 and 53, between positions 53 and 54, between positions 54 and 55, between positions 55 and 56, between positions 155 and 156, between positions 156 and 157, between positions 157 and 158, between positions 158 and 159, between positions 159 and 160, between positions 160 and 161, between positions 187 and 188, between positions 188 and 189, between positions 189 and 190, between positions 217 and 218, or between positions 218 and 219 (BASBPN numbering), in particular between positions 217 and 218.

A subtilase variant of the first aspect of the invention may be a parent or wild-type subtilase identified and isolated from nature.

Such a parent wildtype subtilase may be specifically screened for by standard techniques known in the art.

One preferred way of doing this may be by specifically PCR amplify DNA regions known to encode active site loops in subtilases from numerous different microorganism, preferably different Bacillus strains.

Subtilases are a group of conserved enzymes, in the sense that their DNA and amino acid sequences are homologous. Accordingly it is possible to construct relatively specific primers flanking active site loops.

One way of doing this is by investigating an alignment of different subtilases (see *e.g.* Siezen et al. *Protein Science* **6** (1997) 501-523). It is from this routine work for a person skilled in the art to construct PCR primers flanking the active site loop corresponding to the active site loop (b) between

amino acid residue 95 to 103 in any of the group I-S1 or I-S2 groups, such as from BLSAVI. Using such PCR primers to amplify DNA from a number of different microorganism, preferably different Bacillus strains, followed by DNA sequencing of said amplified PCR fragments, it will be possible to identify strains which produce subtilases of these groups comprising a longer, as compared to *e.g.* BLSAVI, active site region corresponding to the active site loop region from positions 95 to 103. Having identified the strain and a partial DNA sequence of such a subtilase of interest, it is routine work for a person skilled in the art to complete cloning, expression and purification of such a subtilase.

variant. However, it is envisaged that a subtilase parent subtilase. invention predominantly is a variant of a

A subtilase variant suitable for the uses described herein, may be constructed by standard techniques known in the art such as by site-directed/random mutagenesis or by DNA shuffling of different subtilase sequences. See the "Material and Methods" section herein (*vide infra*) for further details.

As will be acknowledged by the skilled person, the variants described herein may comprise one or more further modifications, in particular one or more further substitutions.

Moreover, the insertions in the regions described herein may encompass insertion of more than just one amino acid residue. For example the variant according to the invention may contain one insertion, two insertions, or more than two insertions, such as three, four or five insertions.

In one interesting embodiment of the invention the additional amino acid residue is inserted between positions 42 and 43.

The insertion between positions 42 and 43 is preferably selected from the group consisting of (in BASBPN numbering)

X42X{A,T,G,S}, e.g., X42XA, X42XT, X42XG, X42XS;
X42X{D,E,K,R}, e.g., X42XD, X42XE, X42XK, X42XR;
X42X{H,V,C,N,Q}, e.g., X42XH, X42XV, X42XC, X42XN, X42XQ; and
X42X{F,I,L,M,P,W,Y}, e.g., X42XF, X42XI, X42XL, X42XM, X42XP, X42XW, X42XY;

or more specific for subtilisin 309 and closely related subtilases, such as BAALKP, BLSUBL, and BSKSMK:

D42D{A,T,G,S}, e.g., D42DA, D42DT, D42DG, D42DS;
D42D{D,E,K,R}, e.g., D42DD, D42DE, D42DK, D42DR;
D42D{H,V,C,N,Q}, e.g., D42DH, D42DV, D42DC, D42DN, D42DQ; and
D42D{F,I,L,M,P,W,Y}, e.g., D42DF, D42DI, D42DL, D42DM, D42DP, D42DW, D42DY.

In a further interesting embodiment of the invention the additional amino acid residue is inserted between positions 51 and 52.

The insertion between positions 51 and 52 is preferably selected from the group consisting of (in BASBPN numbering)

X51X{A,T,G,S}, e.g., X51XA, X51XT, X51XG, X51XS;
X51X{D,E,K,R}, e.g., X51XD, X51XE, X51XK, X51XR;
X51X{H,V,C,N,Q}, e.g., X51XH, X51XV, X51XC, X51XN, X51XQ; and
X51X{F,I,L,M,P,W,Y}, e.g., X51XF, X51XI, X51XL, X51XM, X51XP, X51XW, X51XY;

or more specific for subtilisin 309 and closely related subtilases, such as BAALKP, BLSUBL, and BSKSMK:

V51V{A,T,G,S}, e.g., V51VA, V51VT, V51VG, V51VS;
V51V{D,E,K,R}, e.g., V51VD, V51VE, V51VK, V51VR;
V51V{H,V,C,N,Q}, e.g., V51VH, V51VV, V51VC, V51VN, V51VQ; and
V51V{F,I,L,M,P,W,Y}, e.g., V51VF, V51VI, V51VL, V51VM, V51VP, V51VW, V51VY.

In another interesting embodiment of the invention the additional amino acid residue is inserted between positions 52 and 53.

The insertion between positions 52 and 53 is preferably selected from the group consisting of (in BASBPN numbering)

X52X{A,T,G,S}, e.g., X52XA, X52XT, X52XG, X52XS;
X52X{D,E,K,R}, e.g., X52XD, X52XE, X52XK, X52XR;
X52X{H,V,C,N,Q}, e.g., X52XH, X52XV, X52XC, X52XN, X52XQ; and
X52X{F,I,L,M,P,W,Y}, e.g., X52XF, X52XI, X52XL, X52XM, X52XP, X52XW, X52XY;

or more specific for subtilisin 309 and closely related subtilases, such as BAALKP, BLSUBL, and BSKSMK:

P52P{A,T,G,S}, e.g., P52PA, P52PT, P52PG, P52PS;
P52P{D,E,K,R}, e.g., P52PD, P52PE, P52PK, P52PR;
P52P{H,V,C,N,Q}, e.g., P52PH, P52PV, P52PC, P52PN, P52PQ; and
P52P{F,I,L,M,P,W,Y}, e.g., P52PF, P52PI, P52PL, P52PM, P52PP, P52PW, P52PY.

In further interesting embodiment of the invention the additional amino acid residue is inserted between positions 53 and 54.

The insertion between positions 53 and 54 is preferably selected from the group consisting of (in BASBPN numbering)

X53X{A,T,G,S}, e.g., X53XA, X53XT, X53XG, X53XS;
X53X{D,E,K,R}, e.g., X53XD, X53XE, X53XK, X53XR;
X53X{H,V,C,N,Q}, e.g., X53XH, X53XV, X53XC, X53XN, X53XQ; and
X53X{F,I,L,M,P,W,Y}, e.g., X53XF, X53XI, X53XL, X53XM, X53XP, X53XW, X53XY;

or more specific for subtilisin 309 and closely related subtilases, such as BAALKP, BLSUBL, and BSKSMK:

G53G{A,T,G,S}, e.g., G53GA, G53GT, G53GG, G53GS;
G53G{D,E,K,R}, e.g., G53GD, G53GE, G53GK, G53GR;
G53G{H,V,C,N,Q}, e.g., G53GH, G53GV, G53GC, G53GN, G53GQ; and
G53G{F,I,L,M,P,W,Y}, e.g., G53GF, G53GI, G53GL, G53GM, G53GP, G53GW, G53GY.

In a still further interesting embodiment of the invention the additional amino acid residue is inserted between positions 54 and 55.

The insertion between positions 54 and 55 is preferably selected from the group consisting of (in BASBPN numbering)

X54X{A,T,G,S}, e.g., X54XA, X54XT, X54XG, X54XS;
X54X{D,E,K,R}, e.g., X54XD, X54XE, X54XK, X54XR;
X54X{H,V,C,N,Q}, e.g., X54XH, X54XV, X54XC, X54XN, X54XQ; and
X54X{F,I,L,M,P,W,Y}, e.g., X54XF, X54XI, X54XL, X54XM, X54XP, X54XW, X54XY;

or more specific for subtilisin 309 and closely related subtilases, such as BAALKP, BLSUBL, and BSKSMK:

E54E{A,T,G,S}, e.g., E54EA, E54ET, E54EG, E54ES;
E54E{D,E,K,R}, e.g., E54ED, E54EE, E54EK, E54ER;
E54E{H,V,C,N,Q}, e.g., E54EH, E54EV, E54EC, E54EN, E54EQ; and
E54E{F,I,L,M,P,W,Y}, e.g., E54EF, E54EI, E54EL, E54EM, E54EP, E54EW, E54EY.

In an even further interesting embodiment of the invention the additional amino acid residue is inserted between positions 55 and 56.

The insertion between positions 55 and 56 is preferably selected from the group consisting of (in BASBPN numbering)

X55X{A,T,G,S}, e.g., X55XA, X55XT, X55XG, X55XS;
X55X{D,E,K,R}, e.g., X55XD, X55XE, X55XK, X55XR;
X55X{H,V,C,N,Q}, e.g., X55XH, X55XV, X55XC, X55XN, X55XQ; and
X55X{F,I,L,M,P,W,Y}, e.g., X55XF, X55XI, X55XL, X55XM, X55XP, X55XW, X55XY;

or more specific for subtilisin 309 and closely related subtilases, such as BAALKP, BLSUBL, and BSKSMK:

P55P{A,T,G,S}, e.g., P55PA, P55PT, P55PG, P55PS;
P55P{D,E,K,R}, e.g., P55PD, P55PE, P55PK, P55PR;
P55P{H,V,C,N,Q}, e.g., P55PH, P55PV, P55PC, P55PN, P55PQ; and
P55P{F,I,L,M,P,W,Y}, e.g., P55PF, P55PI, P55PL, P55PM, P55PP, P55PW, P55PY.

In another interesting embodiment of the invention the additional amino acid residue is inserted between positions 155 and 156.

The insertion between positions 155 and 156 is preferably selected from the group consisting of (in BASBPN numbering)

X155X{A,T,G,S}, e.g., X155XA, X155XT, X155XG, X155XS;
X155X{D,E,K,R}, e.g., X155XD, X155XE, X155XK, X155XR;
X155X{H,V,C,N,Q}, e.g., X155XH, X155XV, X155XC, X155XN, X155XQ; and
X155X{F,I,L,M,P,W,Y}, e.g., X155XF, X155XI, X155XL, X155XM, X155XP, X155XW, X155XY;

or more specific for subtilisin 309 and closely related subtilases, such as BAALKP, BLSUBL, and BSKSMK:

N155N{A,T,G,S}, e.g., N155NA, N155NT, N155NG, N155NS;
N155N{D,E,K,R}, e.g., N155ND, N155NE, N155NK, N155NR;
N155N{H,V,C,N,Q}, e.g., N155NH, N155NV, N155NC, N155NN, N155NQ; and
N155N{F,I,L,M,P,W,Y}, e.g., N155NF, N155NI, N155NL, N155NM, N155NP, N155NW, N155NY.

In a further interesting embodiment of the invention the additional amino acid residue is inserted between positions 156 and 157.

The insertion between positions 156 and 157 is preferably selected from the group consisting of (in BASBPN numbering)

X156X{A,T,G,S}, e.g., X156XA, X156XT, X156XG, X156XS;
X156X{D,E,K,R}, e.g., X156XD, X156XE, X156XK, X156XR;
X156X{H,V,C,N,Q}, e.g., X156XH, X156XV, X156XC, X156XN, X156XQ; and
X156X{F,I,L,M,P,W,Y}, e.g., X156XF, X156XI, X156XL, X156XM, X156XP, X156XW, X156XY;

or more specific for subtilisin 309 and closely related subtilases, such as BAALKP, BLSUBL, and BSKSMK:

S156S{A,T,G,S}, e.g., S156SA, S156ST, S156SG, S156SS;
S156S{D,E,K,R}, e.g., S156SD, S156SE, S156SK, S156SR;
S156S{H,V,C,N,Q}, e.g., S156SH, S156SV, S156SC, S156SN, S156SQ; and
S156S{F,I,L,M,P,W,Y}, e.g., S156SF, S156SI, S156SL, S156SM, S156SP, S156SW, S156SY.

In a still further interesting embodiment of the invention the additional amino acid residue is inserted between positions 157 and 158.

The insertion between positions 157 and 158 is preferably selected from the group consisting of (in BASBPN numbering)

X157X{A,T,G,S}, e.g., X157XA, X157XT, X157XG, X157XS;
X157X{D,E,K,R}, e.g., X157XD, X157XE, X157XK, X157XR;
X157X{H,V,C,N,Q}, e.g., X157XH, X157XV, X157XC, X157XN, X157XQ; and
X157X{F,I,L,M,P,W,Y}, e.g., X157XF, X157XI, X157XL, X157XM, X157XP, X157XW, X157XY;

or more specific for subtilisin 309 and closely related subtilases, such as BAALKP, BLSUBL, and BSKSMK:

G157G{A,T,G,S}, e.g., G157GA, G157GT, G157GG, G157GS;
G157G{D,E,K,R}, e.g., G157GD, G157GE, G157GK, G157GR;
G157G{H,V,C,N,Q}, e.g., G157GH, G157GV, G157GC, G157GN, G157GQ; and
G157G{F,I,L,M,P,W,Y}, e.g., G157GF, G157GI, G157GL, G157GM, G157GP, G157GW, G157GY.

In an even further interesting embodiment of the invention the additional amino acid residue is inserted between positions 158 and 159.

The insertion between positions 158 and 159 is preferably selected from the group consisting of (in BASBPN numbering)

X158X{A,T,G,S}, e.g., X158XA, X158XT, X158XG, X158XS;
X158X{D,E,K,R}, e.g., X158XD, X158XE, X158XK, X158XR;
X158X{H,V,C,N,Q}, e.g., X158XH, X158XV, X158XC, X158XN, X158XQ; and
X158X{F,I,L,M,P,W,Y}, e.g., X158XF, X158XI, X158XL, X158XM, X158XP, X158XW, X158XY;

or more specific for subtilisin 309 and closely related subtilases, such as BAALKP, BLSUBL, and BSKSMK:

*158{A,T,G,S}, e.g., *158A, *158T, *158G, *158S;
*158{D,E,K,R}, e.g., *158D, *158E, *158K, *158R;
*158{H,V,C,N,Q}, e.g., *158H, *158V, *158C, *158N, *158Q; and
*158{F,I,L,M,P,W,Y}, e.g., *158F, *518I, *158L, *158M, *158P, *158W, *158Y.

In still another interesting embodiment of the invention the additional amino acid residue is inserted between positions 159 and 160.

The insertion between positions 159 and 160 is preferably selected from the group consisting of (in BASBPN numbering)

X159X{A,T,G,S}, e.g., X159XA, X159XT, X159XG, X159XS;
X159X{D,E,K,R}, e.g., X159XD, X159XE, X159XK, X159XR;
X159X{H,V,C,N,Q}, e.g., X159XH, X159XV, X159XC, X159XN, X159XQ; and

X159X{F,I,L,M,P,W,Y}, e.g., X159XF, X159XI, X159XL, X159XM, X159XP, X159XW, X159XY;

or more specific for subtilisin 309 and closely related subtilases, such as BAALKP, BLSUBL, and BSKSMK:

A159A{A,T,G,S}, e.g., A159AA, A159AT, A159AG, A159AS;
A159A{D,E,K,R}, e.g., A159AD, A159AE, A159AK, A159AR;
A159A{H,V,C,N,Q}, e.g., A159AH, A159AV, A159AC, A159AN, A159AQ; and
A159A{F,I,L,M,P,W,Y}, e.g., A159AF, A159AI, A159AL, A159AM, A159AP, A159AW, A159AY.

In a still another interesting embodiment of the invention the additional amino acid residue is inserted between positions 160 and 161.

The insertion between positions 160 and 161 is preferably selected from the group consisting of (in BASBPN numbering)

X160X{A,T,G,S}, e.g., X160XA, X160XT, X160XG, X160XS;
X160X{D,E,K,R}, e.g., X160XD, X160XE, X160XK, X160XR;
X160X{H,V,C,N,Q}, e.g., X160XH, X160XV, X160XC, X160XN, X160XQ; and
X160X{F,I,L,M,P,W,Y}, e.g., X160XF, X160XI, X160XL, X160XM, X160XP, X160XW, X160XY;

or more specific for subtilisin 309 and closely related subtilases, such as BAALKP, BLSUBL, and BSKSMK:

G160G{A,T,G,S}, e.g., G160GA, G160GT, G160GG, G160GS;
G160G{D,E,K,R}, e.g., G160GD, G160GE, G160GK, G160GR;
G160G{H,V,C,N,Q}, e.g., G160GH, G160GV, G160GC, G160GN, G160GQ; and

G160G{F,I,L,M,P,W,Y}, e.g., G160GF, G160GI, G160GL, G160GM, G160GP, G160GW, G160GY.

In another interesting embodiment of the invention the additional amino acid residue is inserted between positions 187 and 188.

The insertion between positions 187 and 188 is preferably selected from the group consisting of (in BASBPN numbering)

X187X{A,T,G,S}, e.g., X187XA, X187XT, X187XG, X187XS;
X187X{D,E,K,R}, e.g., X187XD, X187XE, X187XK, X187XR;
X187X{H,V,C,N,Q}, e.g., X187XH, X187XV, X187XC, X187XN, X187XQ; and
X187X{F,I,L,M,P,W,Y}, e.g., X187XF, X187XI, X187XL, X187XM, X187XP, X187XW, X187XY;

or more specific for subtilisin 309 and closely related subtilases, such as BAALKP, BLSUBL, and BSKSMK:

A187A{A,T,G,S}, e.g., A187AA, A187AT, A187AG, A187AS;
A187A{D,E,K,R}, e.g., A187AD, A187AE, A187AK, A187AR;
A187A{H,V,C,N,Q}, e.g., A187AH, A187AV, A187AC, A187AN, A187AQ; and
A187A{F,I,L,M,P,W,Y}, e.g., A187AF, A187AI, A187AL, A187AM, A187AP, A187AW, A187AY.

In a further interesting embodiment of the invention the additional amino acid residue is inserted between positions 188 and 189.

The insertion between positions 188 and 189 is preferably selected from the group consisting of (in BASBPN numbering)

X188X{A,T,G,S}, e.g., X188XA, X188XT, X188XG, X188XS;
X188X{D,E,K,R}, e.g., X188XD, X188XE, X188XK, X188XR;
X188X{H,V,C,N,Q}, e.g., X188XH, X188XV, X188XC, X188XN, X188XQ; and
X188X{F,I,L,M,P,W,Y}, e.g., X188XF, X188XI, X188XL, X188XM, X188XP, X188XW, X188XY;

or more specific for subtilisin 309 and closely related subtilases, such as BAALKP, BLSUBL, and BSKSMK:

S188S{A,T,G,S}, e.g., S188SA, S188ST, S188SG, S188SS;
S188S{D,E,K,R}, e.g., S188SD, S188SE, S188SK, S188SR;
S188S{H,V,C,N,Q}, e.g., S188SH, S188SV, S188SC, S188SN, S188SQ; and
S188S{F,I,L,M,P,W,Y}, e.g., S188SF, S188SI, S188SL, S188SM, S188SP, S188SW, S188SY.

In a still further interesting embodiment of the invention the additional amino acid residue is inserted between positions 189 and 190.

The insertion between positions 189 and 190 is preferably selected from the group consisting of (in BASBPN numbering)

X189X{A,T,G,S}, e.g., X189XA, X189XT, X189XG, X189XS;
X189X{D,E,K,R}, e.g., X189XD, X189XE, X189XK, X189XR;
X189X{H,V,C,N,Q}, e.g., X189XH, X189XV, X189XC, X189XN, X189XQ; and
X189X{F,I,L,M,P,W,Y}, e.g., X189XF, X189XI, X189XL, X189XM, X189XP, X189XW, X189XY;

or more specific for subtilisin 309 and closely related subtilases, such as BAALKP, BLSUBL, and BSKSMK:

F189F{A,T,G,S}, e.g., F189FA, F189FT, F189FG, F189FS;
F189F{D,E,K,R}, e.g., F189FD, F189FE, F189FK, F189FR;
F189F{H,V,C,N,Q}, e.g., F189FH, F189FV, F189FC, F189FN, F189FQ; and
F189F{F,I,L,M,P,W,Y}, e.g., F189FF, F189FI, F189FL, F189FM, F189FP, F189FW, F189FY.

In another interesting embodiment of the invention the additional amino acid residue is inserted between positions 216 and 217.

The insertion between positions 216 and 217 is preferably selected from the group consisting of (in BASBPN numbering)

X216X{A,T,G,S}, e.g., X216XA, X216XT, X216XG, X216XS;
X216X{D,E,K,R}, e.g., X216XD, X216XE, X216XK, X216XR;
X216X{H,V,C,N,Q}, e.g., X216XH, X216XV, X216XC, X216XN, X216XQ; and
X216X{F,I,L,M,P,W,Y}, e.g., X216XF, X216XI, X216XL, X216XM, X216XP, X216XW, X216XY;

or more specific for subtilisin 309 and closely related subtilases, such as BAALKP, BLSUBL, and BSKSMK:

S216S{A,T,G,S}, e.g., S216SA, S216ST, S216SG, S216SS;
S216S{D,E,K,R}, e.g., S216SD, S216SE, S216SK, S216SR;
S216S{H,V,C,N,Q}, e.g., S216SH, S216SV, S216SC, S216SN, S216SQ; and
S216S{F,I,L,M,P,W,Y}, e.g., S216SF, S216SI, S216SL, S216SM, S216SP, S216SW, S216SY.

In another interesting embodiment of the invention the additional amino acid residue is inserted between positions 217 and 218.

The insertion between positions 217 and 218 is preferably selected from the group consisting of (in BASBPN numbering)

X217X{A,T,G,S}, e.g., X217XA, X217XT, X217XG, X217XS;
X217X{D,E,K,R}, e.g., X217XD, X217XE, X217XK, X217XR;
X217X{H,V,C,N,Q}, e.g., X217XH, X217XV, X217XC, X217XN, X217XQ; and
X217X{F,I,L,M,P,W,Y}, e.g., X217XF, X217XI, X217XL, X217XM, X217XP, X217XW, X217XY;

or more specific for subtilisin 309 and closely related subtilases, such as BAALKP, BLSUBL, and BSKSMK:

L217L{A,T,G,S}, e.g., L217LA, L217LT, L217LG, L217LS;
L217L{D,E,K,R}, e.g., L217LD, L217LE, L217LK, L217LR;
L217L{H,V,C,N,Q}, e.g., L217LH, L217LV, L217LC, L217LN, L217LQ; and
L217L{F,I,L,M,P,W,Y}, e.g., L217LF, L217LI, L217LL, L217LM, L217LP, L217LW, L217LY.

In still another interesting embodiment of the invention the additional amino acid residue is inserted between positions 218 and 219.

The insertion between positions 218 and 219 is preferably selected from the group consisting of (in BASBPN numbering)

X218X{A,T,G,S}, e.g., X218XA, X218XT, X218XG, X218XS;
X218X{D,E,K,R}, e.g., X218XD, X218XE, X218XK, X218XR;
X218X{H,V,C,N,Q}, e.g., X218XH, X218XV, X218XC, X218XN, X218XQ; and
X218X{F,I,L,M,P,W,Y}, e.g., X218XF, X218XI, X218XL, X218XM, X218XP, X218XW, X218XY;

or more specific for subtilisin 309 and closely related subtilases, such as BAALKP, BLSUBL, and BSKSMK:

N218N{A,T,G,S}, e.g., N218NA, N218NT, N218NG, N218NS;
N218N{D,E,K,R}, e.g., N218ND, N218NE, N218NK, N218NR;
N218N{H,V,C,N,Q}, e.g., N218NH, N218NV, N218NC, N218NN, N218NQ; and
N218N{F,I,L,M,P,W,Y}, e.g., N218NF, N218NI, N218NL, N218NM, N218NP, N218NW, N218NY.

Moreover, it is contemplated that, in addition to the above-mentioned insertions performed in accordance with the invention, insertion of at least one additional amino acid residue in the active site loop (b) region from position 95 to 103 (BASBPN numbering) will further reduce the tendency towards inhibition by trypsin inhibitor type IV-0. It is envisaged that additional insertions between position 98 and 99 and/or insertions between positions 99 and 100 will be particular beneficial. Examples of such additional insertions are:

X98X{A,T,G,S}, e.g., X98XA, X98XT, X98XG, X98XS;
X98X{D,E,K,R}, e.g., X98XD, X98XE, X98XK, X98XR;
X98X{H,V,C,N,Q}, e.g., X98XH, X98XV, X98XC, X98XN, X98XQ; and
X98X{F,I,L,M,P,W,Y}, e.g., X98XF, X98XI, X98XL, X98XM, X98XP, X98XW, X98XY; preferably X98XD and X98XE;

or more specific for subtilisin 309 and closely related subtilases:

A98A{A,T,G,S}, e.g., A98AA, A98AT, A98AG, A98AS;
A98A{D,E,K,R}, e.g., A98AD, A98AE, A98AK, A98AR;
A98A{H,V,C,N,Q}, e.g., A98AH, A98AV, A98AC, A98AN, A98AQ;

A98A{F,I,L,M,P,W,Y}, e.g., A98AF, A98AI, A98AL, A98AM, A98AP, A98AW, A98AY; preferably A98AD and A98AE.

Further examples include:

X99X{A,T,G,S}, e.g., X99XA, X99XT, X99XG, X99XS;
X99X{D,E,K,R}, e.g., X99XD, X99XE, X99XK, X99XR;
X99X{H,V,C,N,Q}, e.g., X99XH, X99XV, X99XC, X99XN, X99XQ; and
X99X{F,I,L,M,P,W,Y}, e.g., X99XF, X99XI, X99XL, X99XM, X99XP, X99XW, X99XY; preferably X99XD and X99XE;

or more specific for subtilisin 309 and closely related subtilases:

S99S{A,T,G,S}, e.g., S99SA, S99ST, S99SG, S99SS;
S99S{D,E,K,R}, e.g., S99SD, S99SE, S99SK, S99SR;
S99S{H,V,C,N,Q}, e.g., S99SH, S99SV, S99SC, S99SN, S99SQ;
S99S{F,I,L,M,P,W,Y}, e.g., S99SF, S99SI, S99SL, S99SM, S99SP, S99SW, S99SY; preferably S99SD and S99SE.

With respect to insertions between position 99 and 100, it is preferred that the insertion is combined with a substitution in position 99. Thus, in addition to the contemplated insertions mentioned above, the following substitutions in position 99 are considered relevant:

X99{A,T,G,S}, e.g., X99A, X99T, X99G, X99S;
X99{D,E,K,R}, e.g., X99D, X99E, X99K, X99R;
X99{H,V,C,N,Q}, e.g., X99H, X99V, X99C, X99N, X99Q, and
X99{F,I,L,M,P,W,Y} e.g., X99F, X99I, X99L, X99M, X99P, X99W, X99Y;

or more specific for subtilisin 309 and closely related subtilases:

S99{A,T,G}, e.g., S99A, S99T, S99G;
S99{D,E,K,R}, e.g., S99D, S99E, S99K, S99R;
S99{H,V,C,N,Q}, e.g., S99H, S99V, S99C, S99N, S99Q; and
S99{F,I,L,M,P,W,Y}, e.g., S99F, S99I, S99L, S99M, S99P, S99W, S99SY.

In a preferred embodiment the substitution in position 99 is selected from the group consisting of X99{A,T,G,S}, in particular X99A, or more specific for subtilisin 309 and closely related subtilases: S99{A,T,G}, in particular S99A.

It is well known in the art that a so-called conservative substitution of one amino acid residue to a similar amino acid residue is expected to produce only a minor change in the characteristic of the enzyme.

Table I below list groups of conservative amino acid substitutions.

Table I

Conservative amino acid substitutions

Common Property	Amino Acid
Basic (positive charge)	K = lysine
	H = histidine
Acidic (negative charge)	E = glutamic acid
	D = aspartic acid
Polar	Q = glutamine
	N = asparagine
Hydrophobic	L = leucine
	I = isoleucine
	V = valine
	M = methionine
Aromatic	F = phenylalanine
	W = tryptophan
	Y = tyrosine
Small	G = glycine
	A = alanine
	S = serine
	T = threonine

According to this principle subtilase variants comprising conservative substitutions are expected to exhibit characteristics that are not drastically different from each other.

Based on the disclosed and/or exemplified subtilase variants herein, it is routine work for a person skilled in the art to identify suitable conservative modification(s) to these variants in order to obtain other subtilase variants exhibiting similarly improved wash-performance.

It is preferred that the parent subtilase belongs to the subgroups I-S1 and I-S2, especially subgroup I-S2, both for isolating enzymes from nature or from the artificial creation of diversity, and for designing and producing variants from a parent subtilase.

In relation to variants from subgroup I-S1, it is preferred to select a parent subtilase from the group consisting of BSS168 (BSSAS, BSAPRJ, BSAPRN, BMSAMP), BASBPN, BSSDY, BLSCAR (BLKERA, BLSCA1, BLSCA2, BLSCA3), BSSPRC, and BSSPRD, or functional variants thereof having retained the characteristic of sub-group I-S1.

In relation to variants from subgroup I-S2 it is preferred to select a parent subtilase from the group consisting of BSAPRQ, BLS147 (BSAPRM, BAH101), BLSAVI (BSKSMK, BAALKP, BLSUBL), BYSYAB, BAPB92, TVTHER, and BSAPRS, or functional variants thereof having retained the characteristic of sub-group I-S2. In particular, the parent subtilase is BLSAVI (savinase®, NOVO NORDISK A/S), and a preferred subtilase variant of the invention is accordingly a variant of Savinase®.

The present invention also encompasses any of the above mentioned subtilase variants in combination with any other modification to the amino acid sequence thereof. Especially combinations with other modifications known in the art to provide improved properties to the enzyme are envisaged. The art describes a number of subtilase variants with different improved properties and a number of those are mentioned in the "Background of the invention" section herein (*vide supra*). Those references are disclosed here as references to identify a subtilase variant, which advantageously can be combined with a subtilase variant described herein.

Such combinations comprise the positions: 222 (improves oxidation stability), 218 (improves thermal stability), substitutions in the Ca-binding sites stabilizing the enzyme, *e.g.* position 76, and many other apparent from the prior art.

In further embodiments a subtilase variant described herein may advantageously be combined with one or more modification(s) in any of the positions:

27, 36, 56, 76, 87, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 120, 123, 159, 167, 170, 206, 218, 222, 224, 232, 235, 236, 245, 248, 252 and 274.

Specifically, the following BLSAVI, BLSUBL, BSKSMK, and BAALKP variants are considered appropriate for combination:

K27R, *36D, S56P, N76D, S87N, G97N, S101G, S103A, V104A, V104I, V104N, V104Y, H120D, N123S, G159D, Y167, R170, Q206E, N218S, M222S, M222A, T224S, A232V, K235L, Q236H, Q245R, N248D, N252K and T274A.

Furthermore variants comprising any of the variants S101G+V104N, S87N+S101G+V104N, K27R+V104Y+N123S+T274A, N76D+S103A+V104I or N76D+V104A or other combinations of these mutations (V104N, S101G, K27R, V104Y, N123S, T274A, N76D, V104A) in combination with any one or more of the modification(s) mentioned above exhibit improved properties.

A particular interesting variant is a variant, which, in addition to the insertions according to the invention, contains the following substitutions: S101G+S103A+V104I+G159D+A232V+Q236H+Q245R+N248D+N252K.

Moreover, subtilase variants of the main aspect(s) of the invention are preferably combined with one or more modification(s) in any of the positions 129, 131 and 194, preferably as 129K, 131H and 194P modifications, and most preferably as P129K, P131H and A194P modifications. Any of those modification(s) are expected to provide a higher expression level of the subtilase variant in the production thereof.

As mentioned above, the variants of the invention are only inhibited by trypsin inhibitor type IV-0 to a limited extent and, consequently, they exhibit excellent wash performance on egg stains. Therefore, in order to enable the skilled person - at an early stage of his development work - to select effective and preferred variants for this purpose, the present inventors have provided a suitable preliminary test, which can easily be carried out by the skilled person in order to initially assess the performance of the variant in question.

Thus, the "Ovo-inhibition Assay" disclosed in Example 4 herein may be employed to initially assess the potential of a selected variant. In other words, the "Ovo-inhibition Assay" may be employed to assess whether a selected variant will be inhibited, and to what extent, by the trypsin inhibitor type IV-0. Using this test, the suitability of a selected variant to remove egg stains can be assessed, the rationale being that if a selected variant is strongly inhibited by trypsin inhibitor type IV-0, it is normally not necessary to carry out further test experiments.

Therefore, a variant which is particular interesting for the purposes described herein, is a variant which - when tested in the "Ovo-inhibition Assay" described in Example 4 herein - has a Residual Activity of at least 15%, such as at least 20%, preferably at least 25%, such as at least 30%, more preferably

at least 35%. In a particular interesting embodiment of the invention, the variant has a Residual Activity of at least 40%, such as at least 45%, e.g. at least 50%, preferably at least 55%, such as at least 60%, more preferably at least 65%, such as at least 70%, even more preferably at least 75%, such as at least 80%, e.g. at least 90%, when tested in the "Ovo-inhibition Assay" described in Example 4 herein.

Evidently, it is preferred that the variant of the invention fulfils the above criteria on at least the stated lowest level, more preferably at the stated intermediate level and most preferably on the stated highest level.

Alternatively, or in addition to the above-mentioned assay, the suitability of a selected variant may be tested in the "Model Detergent Wash Performance Test" disclosed in Example 3 herein. The "Model Detergent Wash Perfomance Test" may be employed to assess the ability of a variant, when incorporated in a standard detergent composition, to remove egg stains from a standard textile as compared to a reference system, namely the parent subtilase (incorporated in the same model detergent system and tested under identical conditions). Using this test, the suitability of a selected variant to remove egg stains can be initially investigated, the rationale being that if a selected variant does not show a significant improvement in the test compared to the parent subtilase, it is normally not necessary to carry out further test experiments.

Therefore, variants which are particular interesting for the purposes described herein, are such variants which, when tested in a model detergent composition comprising

6.2% LAS (Nansa 80S)
2% Sodium salt of C_{16}-C_{18} fatty acid

4%	Non-ionic surfactant (Plurafax LF404)
22%	Zeolite P
10.5%	Na_2CO_3
4%	$Na_2Si_2O_5$
2%	Carboxymethylcellulose (CMC)
6.8%	Acrylate liquid CP5 40%
20%	Sodium perborate (empirical formula $NaBO_2.H_2O_2$)
0.2%	EDTA
21%	Na_2SO_4
	Water (balance)

as described in the "Model Detergent Wash Performance Test" herein, shows an improved wash performance on egg stains as compared to the parent subtilase tested under identical conditions.

The improvement in the wash performance may be quantified by employing the so-called "Performance Factor" defined in Example 3, herein.

In a very interesting embodiment of the invention, the variant of the invention, when tested in the "Wash Performance Test" has a Performance Factor of at least 1, such as at least 1.5, e.g. at least 2, preferably at least 2.5, such as at least 3, e.g. at least 3.5, in particular at least 4, such as at least 4.5, e.g. at least 5.

Evidently, it is preferred that the variant of the invention fulfils the above criteria on at least the stated lowest level, more preferably at the stated intermediate level and most preferably on the stated highest level.

PRODUCING A SUBTILASE VARIANT

Many methods for cloning a subtilase and for introducing insertions into genes (*e.g.* subtilase genes) are well known in the art, cf. the references cited in the "BACKGROUND OF THE INVENTION" section.

In general standard procedures for cloning of genes and introducing insertions (random and/or site directed) into said genes may be used in order to obtain a subtilase variant of the invention. For further description of suitable techniques reference is made to Examples herein (*vide infra*) and (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY; Ausubel, F. M. et al. (eds.) "Current protocols in Molecular Biology". John Wiley and Sons, 1995; Harwood, C. R., and Cutting, S. M. (eds.) "Molecular Biological Methods for Bacillus". John Wiley and Sons, 1990); and WO 96/34946.

Further, a subtilase variant may be constructed by standard techniques for artificial creation of diversity, such as by DNA shuffling of different subtilase genes (WO 95/22625; Stemmer WPC, Nature 370:389-91 (1994)). DNA shuffling of *e.g.* the gene encoding Savinase® with one or more partial subtilase sequences identified in nature to comprise an active site (b) loop regions longer than the active site (b) loop of Savinase®, will after subsequent screening for improved wash performance variants, provide subtilase variants suitable for the purposes described herein.

EXPRESSION VECTORS

A recombinant expression vector comprising a DNA construct encoding the enzyme of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures.

The choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid.

Alternatively, the vector may be one that on introduction into a host cell is integrated into the host cell genome in part or in its entirety and replicated together with the chromosome(s) into which it has been integrated.

The vector is preferably an expression vector in which the DNA sequence encoding the enzyme of the invention is operably linked to additional segments required for transcription of the DNA. In general, the expression vector is derived from plasmid or viral DNA, or may contain elements of both. The term, "operably linked" indicates that the segments are arranged so that they function in concert for their intended purposes, e.g. transcription initiates in a promoter and proceeds through the DNA sequence coding for the enzyme.

The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell.

Examples of suitable promoters for use in bacterial host cells include the promoter of the *Bacillus stearothermophilus* maltogenic amylase gene, the *Bacillus licheniformis* alpha-amylase gene, the *Bacillus amyloliquefaciens* alpha-amylase gene, the *Bacillus subtilis* alkaline protease gen, or the *Bacillus pumilus* xylosidase gene, or the phage Lambda P_R or P_L promoters or the E. coli lac, trp or tac promoters.

The DNA sequence encoding the enzyme of the invention may also, if necessary, be operably connected to a suitable terminator.

The recombinant vector of the invention may further comprise a DNA sequence enabling the vector to replicate in the host cell in question.

The vector may also comprise a selectable marker, *e.g.* a gene the product of which complements a defect in the host cell, or a gene encoding resistance to e.g. antibiotics like kanamycin, chloramphenicol, erythromycin, tetracycline, spectinomycine, or the like, or resistance to heavy metals or herbicides.

To direct an enzyme of the present invention into the secretory pathway of the host cells, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) may be provided in the recombinant vector. The secretory signal sequence is joined to the DNA sequence encoding the enzyme in the correct reading frame. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the enzyme. The secretory signal sequence may be that normally associated with the enzyme or may be from a gene encoding another secreted protein.

The procedures used to ligate the DNA sequences coding for the present enzyme, the promoter and optionally the terminator and/or secretory signal sequence, respectively, or to assemble these sequences by suitable PCR amplification schemes, and to insert them into suitable vectors containing the information necessary for replication or integration, are well known to persons skilled in the art (cf., for instance, Sambrook et al., op.cit.).

HOST CELL

The DNA sequence encoding the present enzyme introduced into the host cell may be either homologous or heterologous to the host in question. If homologous to the host cell, i.e. produced by the host cell in nature, it will typically be operably connected to another promoter sequence or, if applicable, another secretory signal sequence and/or terminator sequence than in its natural environment. The term "homologous" is intended to include a DNA sequence encoding an enzyme native to the host organism in question. The term "heterologous" is intended to include a DNA sequence not expressed by the host cell in nature. Thus, the DNA sequence may be from another organism, or it may be a synthetic sequence.

The host cell into which the DNA construct or the recombinant vector of the invention is introduced may be any cell which is capable of producing the present enzyme and includes bacteria, yeast, fungi and higher eukaryotic cells including plants.

Examples of bacterial host cells which, on cultivation, are capable of producing the enzyme of the invention are gram-positive bacteria such as strains of *Bacillus*, such as strains of *B. subtilis*, *B. licheniformis*, *B. lentus*, *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. amyloliquefaciens*, *B. coagulans*, *B. circulans*, *B. lautus*, *B. megatherium* or *B. thuringiensis*, or strains of *Streptomyces*, such as *S. lividans* or *S. murinus*, or gram-negative bacteria such as *Echerichia coli*.

The transformation of the bacteria may be effected by protoplast transformation, electroporation, conjugation, or by using competent cells in a manner known per se (cf. Sambrook et al., supra).

When expressing the enzyme in bacteria such as *E. coli*, the enzyme may be retained in the cytoplasm, typically as insoluble granules (known as inclusion bodies), or may be directed to the periplasmic space by a bacterial secretion sequence. In the former case, the cells are lysed and the granules are recovered and denatured after which the enzyme is refolded by diluting the denaturing agent. In the latter case, the enzyme may be recovered from the periplasmic space by disrupting the cells, e.g. by sonication or osmotic shock, to release the contents of the periplasmic space and recovering the enzyme.

When expressing the enzyme in gram-positive bacteria such as Bacillus or Streptomyces strains, the enzyme may be retained in the cytoplasm, or may be directed to the extracellular medium by a bacterial secretion sequence. In the latter case, the enzyme may be recovered from the medium as described below.

METHOD FOR PRODUCING A SUBTILASE VARIANT

The present invention provides a method of producing an isolated enzyme according to the invention, wherein a suitable host cell, which has been transformed with a DNA sequence encoding the enzyme, is cultured under conditions permitting the production of the enzyme, and the resulting enzyme is recovered from the culture.

When an expression vector comprising a DNA sequence encoding the enzyme is transformed into a heterologous host cell it is possible to enable heterologous recombinant production of the enzyme of the invention.

Thereby it is possible to make a highly purified subtilase composition, characterized in being free from homologous impurities.

In this context homologous impurities means any impurities (e.g. other polypeptides than the enzyme of the invention) which originate from the homologous cell where the enzyme of the invention is originally obtained from.

The medium used to culture the transformed host cells may be any conventional medium suitable for growing the host cells in question. The expressed subtilase may conveniently be secreted into the culture medium and may be recovered therefrom by well-known procedures including separating the cells from the medium by centrifugation or filtration, precipitating proteinaceous components of the medium by means of a salt such as ammonium sulfate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

CLEANING AND DETERGENT COMPOSITIONS

In general, cleaning and detergent compositions are well described in the art and reference is made to WO 96/34946; WO 97/07202; WO 95/30011 for further description of suitable cleaning and detergent compositions.

Furthermore the examples herein demonstrate the improvements in wash performance on egg stains for a number of subtilase variants.

Detergent Compositions

The subtilase variant may be added to and thus become a component of a detergent composition.

The detergent composition of the invention may for example be formulated as a hand or machine laundry detergent composition including a laundry additive composition suitable for pre-treatment of stained fabrics and a rinse added fabric softener composition, or be formulated as a detergent composition for

use in general household hard surface cleaning operations, or be formulated for hand or machine dishwashing operations.

In a specific aspect, the invention provides a detergent additive comprising a subtilase enzyme of the invention. The detergent additive as well as the detergent composition may comprise one or more other enzymes such as another protease, a lipase, a cutinase, an amylase, a carbohydrase, a cellulase, a pectinase, a mannanase, an arabinase, a galactanase, a xylanase, an oxidase, e.g., a laccase, and/or a peroxidase.

In general the properties of the chosen enzyme(s) should be compatible with the selected detergent, (i.e. pH-optimum, compatibility with other enzymatic and non-enzymatic ingredients, etc.), and the enzyme(s) should be present in effective amounts.

Proteases: Suitable proteases include those of animal, vegetable or microbial origin. Microbial origin is preferred. Chemically modified or protein engineered mutants are included. The protease may be a serine protease or a metallo protease, preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from *Bacillus*, e.g., subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168 (described in WO 89/06279). Examples of trypsin-like proteases are trypsin (e.g. of porcine or bovine origin) and the *Fusarium* protease described in WO 89/06270 and WO 94/25583.

Examples of useful proteases are the variants described in WO 92/19729, WO 98/20115, WO 98/20116, and WO 98/34946, especially the variants with substitutions in one or more of the following

positions: 27, 36, 57, 76, 87, 97, 101, 104, 120, 123, 167, 170, 194, 206, 218, 222, 224, 235 and 274.

Preferred commercially available protease enzymes include Alcalase™, Savinase™, Primase™, Duralase™, Esperase™, and Kannase™ (Novo Nordisk A/S), Maxatase™, Maxacal™, Maxapem™, Properase™, Purafect™, Purafect OxP™, FN2™, and FN3™ (Genencor International Inc.).

Lipases: Suitable lipases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful lipases include lipases from *Humicola* (synonym *Thermomyces*), e.g. from *H. lanuginosa* (*T. lanuginosus*) as described in EP 258 068 and EP 305 216 or from *H. insolens* as described in WO 96/13580, a *Pseudomonas* lipase, e.g. from *P. alcaligenes* or *P. pseudoalcaligenes* (EP 218 272), *P. cepacia* (EP 331 376), *P. stutzeri* (GB 1,372,034), *P. fluorescens*, *Pseudomonas sp.* strain SD 705 (WO 95/06720 and WO 96/27002), *P. wisconsinensis* (WO 96/12012), a *Bacillus* lipase, e.g. from *B. subtilis* (Dartois et al. (1993), Biochemica et Biophysica Acta, 1131, 253-360), *B. stearothermophilus* (JP 64/744992) or *B. pumilus* (WO 91/16422).

Other examples are lipase variants such as those described in WO 92/05249, WO 94/01541, EP 407 225, EP 260 105, WO 95/35381, WO 96/00292, WO 95/30744, WO 94/25578, WO 95/14783, WO 95/22615, WO 97/04079 and WO 97/07202.

Preferred commercially available lipase enzymes include Lipolase™ and Lipolase Ultra™ (Novo Nordisk A/S).

Amylases: Suitable amylases (α and/or β) include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Amylases include, for example,

α-amylases obtained from *Bacillus*, e.g. a special strain of *B. licheniformis*, described in more detail in GB 1,296,839.

Examples of useful amylases are the variants described in WO 94/02597, WO 94/18314, WO 96/23873, and WO 97/43424, especially the variants with substitutions in one or more of the following positions: 15, 23, 105, 106, 124, 128, 133, 154, 156, 181, 188, 190, 197, 202, 208, 209, 243, 264, 304, 305, 391, 408, and 444.

Commercially available amylases are Duramyl™, Termamyl™, Fungamyl™ and BAN™ (Novo Nordisk A/S), Rapidase™ and Purastar™ (from Genencor International Inc.).

Cellulases: Suitable cellulases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Suitable cellulases include cellulases from the genera *Bacillus, Pseudomonas, Humicola, Fusarium, Thielavia, Acremonium,* e.g. the fungal cellulases produced from *Humicola insolens, Myceliophthora thermophila* and *Fusarium oxysporum* disclosed in US 4,435,307, US 5,648,263, US 5,691,178, US 5,776,757 and WO 89/09259.

Especially suitable cellulases are the alkaline or neutral cellulases having colour care benefits. Examples of such cellulases are cellulases described in EP 0 495 257, EP 0 531 372, WO 96/11262, WO 96/29397, WO 98/08940. Other examples are cellulase variants such as those described in WO 94/07998, EP 0 531 315, US 5,457,046, US 5,686,593, US 5,763,254, WO 95/24471, WO 98/12307 and PCT/DK98/00299.

Commercially available cellulases include Celluzyme™, and Carezyme™ (Novo Nordisk A/S), Clazinase™, and Puradax HA™ (Genencor International Inc.), and KAC-500(B)™ (Kao Corporation).

Peroxidases/Oxidases: Suitable peroxidases/oxidases include those of plant, bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful peroxidases include peroxidases from *Coprinus*, e.g. from *C. cinereus*, and variants thereof as those described in WO 93/24618, WO 95/10602, and WO 98/15257.

Commercially available peroxidases include Guardzyme™ (Novo Nordisk A/S).

The detergent enzyme(s) may be included in a detergent composition by adding separate additives containing one or more enzymes, or by adding a combined additive comprising all of these enzymes. A detergent additive of the invention, i.e. a separate additive or a combined additive, can be formulated e.g. as a granulate, a liquid, a slurry, etc. Preferred detergent additive formulations are granulates, in particular non-dusting granulates, liquids, in particular stabilized liquids, or slurries.

Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molar weights of 1000 to 20000; ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar

or sugar alcohol, lactic acid or boric acid according to established methods. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent composition of the invention may be in any convenient form, e.g., a bar, a tablet, a powder, a granule, a paste or a liquid. A liquid detergent may be aqueous, typically containing up to 70% water and 0-30% organic solvent, or non-aqueous.

The detergent composition typically comprises one or more surfactants, which may be non-ionic including semi-polar and/or anionic and/or cationic and/or zwitterionic. The surfactants are typically present at a level of from 0.1% to 60% by weight. When included therein the detergent will usually contain from about 1% to about 40% of an anionic surfactant such as linear alkylbenzenesulfonate, alpha-olefinsulfonate, alkyl sulfate (fatty alcohol sulfate), alcohol ethoxysulfate, secondary alkanesulfonate, alpha-sulfo fatty acid methyl ester, alkyl- or alkenylsuccinic acid or soap.

When included therein the detergent will usually contain from about 0.2% to about 40% of a non-ionic surfactant such as alcohol ethoxylate, nonylphenol ethoxylate, alkylpolyglycoside, alkyldimethylamineoxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, polyhydroxy alkyl fatty acid amide, or N-acyl N-alkyl derivatives of glucosamine ("glucamides").

The detergent may contain 0-65% of a detergent builder or complexing agent such as zeolite, diphosphate, triphosphate, phosphonate, carbonate, citrate, nitrilotriacetic acid, ethylenediaminetetraacetic acid, diethylenetriaminepentaacetic acid,

alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (e.g. SKS-6 from Hoechst).

The detergent may comprise one or more polymers. Examples are carboxymethylcellulose, poly(vinylpyrrolidone), poly (ethylene glycol), poly(vinyl alcohol), poly(vinylpyridine-N-oxide), poly(vinylimidazole), polycarboxylates such as polyacrylates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid copolymers.

The detergent may contain a bleaching system which may comprise a H_2O_2 source such as perborate or percarbonate which may be combined with a peracid-forming bleach activator such as tetraacetylethylenediamine or nonanoyloxybenzenesulfonate. Alternatively, the bleaching system may comprise peroxyacids of e.g. the amide, imide, or sulfone type.

The enzyme(s) of the detergent composition of the invention may be stabilized using conventional stabilizing agents, e.g., a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative, e.g., an aromatic borate ester, or a phenyl boronic acid derivative such as 4-formylphenyl boronic acid, and the composition may be formulated as described in e.g. WO 92/19709 and WO 92/19708.

The detergent may also contain other conventional detergent ingredients such as e.g. fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil redeposition agents, dyes, bactericides, optical brighteners, hydrotropes, tarnish inhibitors, or perfumes.

It is at present contemplated that in the detergent compositions any enzyme, in particular the enzyme of the invention, may be added in an amount corresponding to 0.01-100 mg of enzyme protein per liter of wash liquor, preferably 0.05-5 mg of enzyme protein per liter of wash liquor, in particular 0.1-1 mg of enzyme protein per liter of wash liquor.

The enzyme of the invention may additionally be incorporated in the detergent formulations disclosed in WO 97/07202 which is hereby incorporated as reference.

The invention is described in further detail in the following examples, which are not in any way intended to limit the scope of the invention as claimed.

In the detergent compositions, the abbreviated component identifications have the following meanings:

LAS: Sodium linear C_{12} alkyl benzene sulphonate

TAS: Sodium tallow alkyl sulphate

XYAS: Sodium C_{1X} - C_{1Y} alkyl sulfate

SS: Secondary soap surfactant of formula 2-butyl octanoic acid

25EY: A C_{12}-C_{15} predominantly linear primary alcohol condensed with an average of Y moles of ethylene oxide

45EY: A C_{14}-C_{15} predominantly linear primary alcohol condensed with an average of Y moles of ethylene oxide

XYEZS: C_{1X}-C_{1Y} sodium alkyl sulfate condensed with an average of Z moles of ethylene oxide per mole

Nonionic: C_{13}-C_{15} mixed ethoxylated/propoxylated fatty alcohol with an average degree of ethoxylation of 3.8 and an average degree of propoxylation of 4.5 sold under the tradename Plurafax LF404 by BASF GmbH

CFAA: C_{12}-C_{14} alkyl N-methyl glucamide

TFAA: C_{16}-C_{18} alkyl N-methyl glucamide

Silicate: Amorphous Sodium Silicate (SiO_2:Na_2O ratio = 2.0)

NaSKS-6: Crystalline layered silicate of formula δ-$Na_2Si_2O_5$

Carbonate: Anhydrous sodium carbonate

Phosphate: Sodium tripolyphosphate

MA/AA: Copolymer of 1:4 maleic/acrylic acid, average molecular weight about 80,000

Polyacrylate: Polyacrylate homopolymer with an average molecular weight of 8,000 sold under the tradename PA30 by BASF Gmbh

Zeolite A: Hydrated Sodium Aluminosilicate of formula $Na_{12}(AlO_2SiO_2)_{12}.27H_2O$ having a primary particle size in the range from 1 to 10 micrometers

Citrate: Tri-sodium citrate dihydrate

Citric: Citric Acid

Perborate: Anhydrous sodium perborate monohydrate bleach, empirical formula $NaBO_2.H_2O_2$

PB4: Anhydrous sodium perborate tetrahydrate

Percarbonate: Anhydrous sodium percarbonate bleach of empirical formula $2Na_2CO_3.3H_2O_2$

TAED: Tetraacetyl ethylene diamine

CMC: Sodium carboxymethyl cellulose

DETPMP: Diethylene triamine penta (methylene phosphonic acid), marketed by Monsanto under the Tradename Dequest 2060

PVP: Polyvinylpyrrolidone polymer

EDDS: Ethylenediamine-N, N'-disuccinic acid, [S,S] isomer in the form of the sodium salt

Suds Suppressor: 25% paraffin wax Mpt 50°C, 17% hydrophobic silica, 58% paraffin oil

Granular Suds suppressor: 12% Silicone/silica, 18% stearyl alcohol, 70% starch in granular form

Sulphate: Anhydrous sodium sulphate

HMWPEO: High molecular weight polyethylene oxide

TAE 25: Tallow alcohol ethoxylate (25)

Detergent Example I

A granular fabric cleaning composition in accordance with the invention may be prepared as follows:

Sodium linear C_{12} alkyl benzene sulfonate	6.5
Sodium sulfate	15.0
Zeolite A	26.0
Sodium nitrilotriacetate	5.0
Enzyme	0.1
PVP	0.5
TAED	3.0
Boric acid	4.0
Perborate	18.0
Phenol sulphonate	0.1
Minors	up to 100%

Detergent Example II

A compact granular fabric cleaning composition (density 800 g/l) in accord with the invention may be prepared as follows:

45AS	8.0
25E3S	2.0
25E5	3.0
25E3	3.0
TFAA	2.5
Zeolite A	17.0
NaSKS-6	12.0
Citric acid	3.0
Carbonate	7.0
MA/AA	5.0
CMC	0.4

Enzyme	0.1
TAED	6.0
Percarbonate	22.0
EDDS	0.3
Granular suds suppressor	3.5
water/minors	Up to 100%

Detergent Example III

Granular fabric cleaning compositions in accordance with the invention which are especially useful in the laundering of coloured fabrics were prepared as follows:

LAS	10.7	-
TAS	2.4	-
TFAA	-	4.0
45AS	3.1	10.0
45E7	4.0	-
25E3S	-	3.0
68E11	1.8	-
25E5	-	8.0
Citrate	15.0	7.0
Carbonate	-	10.0
Citric acid	2.5	3.0
Zeolite A	32.1	25.0
Na-SKS-6	-	9.0
MA/AA	5.0	5.0
DETPMP	0.2	0.8
Enzyme	0.10	0.05
Silicate	2.5	-
Sulphate	5.2	3.0
PVP	0.5	-
Poly (4-vinylpyridine)-N-Oxide/copolymer of vinyl-imidazole and vinyl-	-	0.2

pyrrolidone		
Perborate	1.0	-
Phenol sulfonate	0.2	-
Water/Minors	Up to 100%	

Detergent Example IV

Granular fabric cleaning compositions in accordance with the invention which provide "Softening through the wash" capability may be prepared as follows:

45AS	-	10.0
LAS	7.6	-
68AS	1.3	-
45E7	4.0	-
25E3	-	5.0
Coco-alkyl-dimethyl hydroxy-ethyl ammonium chloride	1.4	1.0
Citrate	5.0	3.0
Na-SKS-6	-	11.0
Zeolite A	15.0	15.0
MA/AA	4.0	4.0
DETPMP	0.4	0.4
Perborate	15.0	-
Percarbonate	-	15.0
TAED	5.0	5.0
Smectite clay	10.0	10.0
HMWPEO	-	0.1
Enzyme	0.10	0.05
Silicate	3.0	5.0
Carbonate	10.0	10.0
Granular suds suppressor	1.0	4.0
CMC	0.2	0.1

Water/Minors Up to 100%

Detergent Example V

Heavy duty liquid fabric cleaning compositions in accordance with the invention may be prepared as follows:

LAS acid form	-	25.0
Citric acid	5.0	2.0
25AS acid form	8.0	-
25AE2S acid form	3.0	-
25AE7	8.0	-
CFAA	5	-
DETPMP	1.0	1.0
Fatty acid	8	-
Oleic acid	-	1.0
Ethanol	4.0	6.0
Propanediol	2.0	6.0
Enzyme	0.10	0.05
Coco-alkyl dimethyl hydroxy ethyl ammonium chloride	-	3.0
Smectite clay	-	5.0
PVP	2.0	-
Water / Minors	Up to 100%	

Powder automatic dishwash composition I

Nonionic surfactant	0.4 - 2.5%
Sodium metasilicate	0 - 20%
Sodium disilicate	3 - 20%
Sodium triphosphate	20 - 40%
Sodium carbonate	0 - 20%

Sodium perborate	2 - 9%
Tetraacetyl ethylene diamine (TAED)	1 - 4%
Sodium sulphate	5 - 33%
Enzymes	0.0001 - 0.1%

Powder automatic dishwash composition II

Nonionic surfactant (e.g. alcohol ethoxylate)	1 - 2%
Sodium disilicate	2 - 30%
Sodium carbonate	10 - 50%
Sodium phosphonate	0 - 5%
Trisodium citrate dihydrate	9 - 30%
Nitrilotrisodium acetate (NTA)	0 - 20%
Sodium perborate monohydrate	5 - 10%
Tetraacetyl ethylene diamine (TAED)	1 - 2%
Polyacrylate polymer (e.g. maleic acid/acrylic acid co-polymer)	6 - 25%
Enzymes	0.0001 - 0.1%
Perfume	0.1 - 0.5%
Water	5 - 10

Powder automatic dishwash composition III

Nonionic surfactant	0.5 - 2.0%
Sodium disilicate	25 - 40%
Sodium citrate	30 - 55%
Sodium carbonate	0 - 29%
Sodium bicarbonate	0 - 20%
Sodium perborate monohydrate	0 - 15%
Tetraacetyl ethylene diamine (TAED)	0 - 6%

Maleic acid/acrylic acid copolymer	0 - 5%
Clay	1 - 3%
Polyamino acids	0 - 20%
Sodium polyacrylate	0 - 8%
Enzymes	0.0001 - 0.1%

Powder automatic dishwash composition IV

Nonionic surfactant	1 - 2%
Zeolite MAP	15 - 42%
Sodium disilicate	30 - 34%
Sodium citrate	0 - 12%
Sodium carbonate	0 - 20%
Sodium perborate monohydrate	7 - 15%
Tetraacetyl ethylene diamine (TAED)	0 - 3%
Polymer	0 - 4%
Maleic acid/acrylic acid copolymer	0 - 5%
Organic phosphonate	0 - 4%
Clay	1 - 2%
Enzymes	0.0001 - 0.1%
Sodium sulphate	Balance

Powder automatic dishwash composition V

Nonionic surfactant	1 - 7%
Sodium disilicate	18 - 30%
Trisodium citrate	10 - 24%
Sodium carbonate	12 - 20%
Monopersulphate (2 $KHSO_5.KHSO_4.K_2SO_4$)	15 - 21%
Bleach stabilizer	0.1 - 2%
Maleic acid/acrylic acid copolymer	0 - 6%

Diethylene triamine pentaacetate, pentasodium salt	0 - 2.5%
Enzymes	0.0001 - 0.1%
Sodium sulphate, water	Balance

Powder and liquid dishwash composition with cleaning surfactant system VI

Nonionic surfactant	0 - 1.5%
Octadecyl dimethylamine N-oxide dihydrate	0 - 5%
80:20 wt.C18/C16 blend of octadecyl dimethylamine N-oxide dihydrate and hexadecyldimethyl amine N-oxide dihydrate	0 - 4%
70:30 wt.C18/C16 blend of octadecyl bis (hydroxyethyl)amine N-oxide anhydrous and hexadecyl bis (hydroxyethyl)amine N-oxide anhydrous	0 - 5%
C_{13}-C_{15} alkyl ethoxysulfate with an average degree of ethoxylation of 3	0 - 10%
C_{12}-C_{15} alkyl ethoxysulfate with an average degree of ethoxylation of 3	0 - 5%
C_{13}-C_{15} ethoxylated alcohol with an average degree of ethoxylation of 12	0 - 5%
A blend of C_{12}-C_{15} ethoxylated alcohols with an average degree of ethoxylation of 9	0 - 6.5%
A blend of C_{13}-C_{15} ethoxylated alcohols with an average degree of ethoxylation of 30	0 - 4%
Sodium disilicate	0 - 33%

Sodium tripolyphosphate	0 - 46%
Sodium citrate	0 - 28%
Citric acid	0 - 29%
Sodium carbonate	0 - 20%
Sodium perborate monohydrate	0 - 11.5%
Tetraacetyl ethylene diamine (TAED)	0 - 4%
Maleic acid/acrylic acid copolymer	0 - 7.5%
Sodium sulphate	0 - 12.5%
Enzymes	0.0001 - 0.1%

Non-aqueous liquid automatic dishwshing composition VII

Liquid nonionic surfactant (e.g. alcohol ethoxylates)	2.0 - 10.0%
Alkali metal silicate	3.0 - 15.0%
Alkali metal phosphate	20.0 - 40.0%
Liquid carrier selected from higher glycols, polyglycols, polyoxides, glycolethers	25.0 - 45.0%
Stabilizer (e.g. a partial ester of phosphoric acid and a C_{16}-C_{18} alkanol)	0.5 - 7.0%
Foam suppressor (e.g. silicone)	0 - 1.5%
Enzymes	0.0001 - 0.1%

Non-aqueous liquid dishwashing composition VIII

Liquid nonionic surfactant (e.g. alcohol ethoxylates)	2.0 - 10.0%
Sodium silicate	3.0 - 15.0%
Alkali metal carbonate	7.0 - 20.0%
Sodium citrate	0.0 - 1.5%
Stabilizing system (e.g. mixtures of	

finely divided silicone and low molecular weight dialkyl polyglycol ethers)	0.5 - 7.0%
Low molecule weight polyacrylate polymer	5.0 - 15.0%
Clay gel thickener (e.g. bentonite)	0.0 - 10.0%
Hydroxypropyl cellulose polymer	0.0 - 0.6%
Enzymes	0.0001 - 0.1%
Liquid carrier selected from higher lycols, polyglycols, polyoxides and glycol ethers	Balance

Thixotropic liquid automatic dishwashing composition IX

C_{12}-C_{14} fatty acid	0 - 0.5%
Block co-polymer surfactant	1.5 - 15.0%
Sodium citrate	0 - 12%
Sodium tripolyphosphate	0 - 15%
Sodium carbonate	0 - 8%
Aluminium tristearate	0 - 0.1%
Sodium cumene sulphonate	0 - 1.7%
Polyacrylate thickener	1.32 - 2.5%
Sodium polyacrylate	2.4 - 6.0%
Boric acid	0 - 4.0%
Sodium formate	0 - 0.45%
Calcium formate	0 - 0.2%
Sodium n-decydiphenyl oxide disulphonate	0 - 4.0%
Monoethanol amine (MEA)	0 - 1.86%
Sodium hydroxide (50%)	1.9 - 9.3%
1,2-Propanediol	0 - 9.4%
Enzymes	0.0001 - 0.1%
Suds suppressor, dye, perfumes, wa-	

ter	Balance

Liquid automatic dishwashing composition X

Alcohol ethoxylate	0 - 20%
Fatty acid ester sulphonate	0 - 30%
Sodium dodecyl sulphate	0 - 20%
Alkyl polyglycoside	0 - 21%
Oleic acid	0 - 10%
Sodium disilicate monohydrate	18 - 33%
Sodium citrate dihydrate	18 - 33%
Sodium stearate	0 - 2.5%
Sodium perborate monohydrate	0 - 13%
Tetraacetyl ethylene diamine (TAED)	0 - 8%
Maleic acid/acrylic acid copolymer	4 - 8%
Enzymes	0.0001 - 0.1%

Liquid automatic dishwashing composition containing protected bleach particles XI

Sodium silicate	5 - 10%
Tetrapotassium pyrophosphate	15 - 25%
Sodium triphosphate	0 - 2%
Potassium carbonate	4 - 8%
Protected bleach particles, e.g. chlorine	5 - 10%
Polymeric thickener	0.7 - 1.5%
Potassium hydroxide	0 - 2%
Enzymes	0.0001 - 0.1%
Water	Balance

XII: Automatic dishwashing compositions as described in I, II, III, IV, VI and X, wherein perborate is replaced by percarbonate.

XIII: Automatic dishwashing compositions as described in I-VI, which additionally contain a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching", *Nature*, (1994), **369**, 637-639.

MATERIALS AND METHODS

TEXTILES:

WFK10N standard textile pieces (egg stains) were obtained from WFK Testgewebe GmbH, Christenfeld 10, D-41379 Brüggen-Bracht, Germany.

STRAINS:

B. subtilis DN1885 (Diderichsen et al., 1990).

B. lentus 309 and 147 are specific strains of *Bacillus lentus*, deposited with the NCIB and accorded the accession numbers NCIB 10309 and 10147, and described in US Patent No. 3,723,250 incorporated by reference herein.

E. coli MC 1000 (M.J. Casadaban and S.N. Cohen (1980); *J. Mol. Biol.* **138** 179-207), was made r^-,m^+ by conventional methods and is also described in US Patent Application Serial No. 039,298.

PLASMIDS:

pJS3: *E. coli* - *B. subtilis* shuttle vector containing a synthetic gene encoding for subtilase 309 (Described by Jacob Schiødt et al. in Protein and Peptide letters **3**:39-44 (1996)).

pSX222: *B. subtilis* expression vector (described in WO 96/34946).

GENERAL MOLECULAR BIOLOGY METHODS:

Unless otherwise mentioned the DNA manipulations and transformations were performed using standard methods of molecular biology (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY; Ausubel, F. M. et al. (eds.) "Current protocols in Molecular Biology". John Wiley and Sons, 1995; Harwood, C. R., and Cutting, S. M. (eds.) "Molecular Biological Methods for Bacillus". John Wiley and Sons, 1990).

Enzymes for DNA manipulations were used according to the specifications of the suppliers.

ENZYMES FOR DNA MANIPULATIONS

Unless otherwise mentioned all enzymes for DNA manipulations, such as *e.g.* restiction endonucleases, ligases etc., are obtained from New England Biolabs, Inc.

PROTEOLYTIC ACTIVITY

In the context of this invention proteolytic activity is expressed in Kilo NOVO Protease Units (KNPU). The activity is determined relatively to an enzyme standard (SAVINASE®), and the determination is based on the digestion of a dimethyl casein (DMC) solution by the proteolytic enzyme at standard conditions, i.e. 50°C, pH 8.3, 9 min. reaction time, 3 min. measuring time. A folder AF 220/1 is available upon request to Novo Nordisk A/S, Denmark, which folder is hereby included by reference.

A GU is a Glycine Unit, defined as the proteolytic enzyme activity which, under standard conditions, during a 15 minutes'

incubation at 40°C, with N-acetyl casein as substrate, produces an amount of NH_2-group equivalent to 1 mmole of glycine.

Enzyme activity can also be measured using the PNA assay, according to reaction with the soluble substrate succinyl-alanine-alanine-proline-phenyl-alanine-para-nitro-phenol, which is described in the Journal of American Oil Chemists Society, Rothgeb, T.M., Goodlander, B.D., Garrison, P.H., and Smith, L.A., (1988).

FERMENTATION:

Fermentations for the production of subtilase enzymes were performed at 30°C on a rotary shaking table (300 r.p.m.) in 500 ml baffled Erlenmeyer flasks containing 100 ml BPX medium for 5 days.

Consequently in order to make an *e.g.* 2 liter broth 20 Erlenmeyer flasks were fermented simultaneously.

MEDIA:

BPX Medium Composition (per liter)

Potato starch	100 g
Ground barley	50 g
Soybean flour	20 g
$Na_2HPO_4 \times 12\ H_2O$	9 g
Pluronic	0.1 g
Sodium caseinate	10 g

The starch in the medium is liquefied with α-amylase and the medium is sterilized by heating at 120°C for 45 minutes. After sterilization the pH of the medium is adjusted to 9 by addition of $NaHCO_3$ to 0.1 M.

EXAMPLE 1

CONSTRUCTION AND EXPRESSION OF ENZYME VARIANTS:

SITE-DIRECTED MUTAGENESIS:

Subtilase 309 (savinase®) site-directed variants of the invention comprising specific insertions and comprising specific substitutions were made by traditional cloning of DNA fragments (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989) produced by PCR with oligos containing the desired insertions (see below).

The template plasmid DNA was pJS3 (see below), or an analogue of this containing a variant of Subtilase 309.

Insertions and substitutions were introduced by oligo directed mutagenesis to the construction of variants.

The Subtilase 309 variants were transformed into *E. coli*. DNA purified from a over night culture of these transformants were transformed into *B. subtilis* by restriction endonuclease digestion, purification of DNA fragments, ligation, transformation of *B. subtilis*. Transformation of B. subtilis was performed as described by Dubnau et al., 1971, J. Mol. Biol. 56, pp. 209-221.

SITE-DIRECTED MUTAGENESIS IN ORDER TO INTRODUCE INSERTIONS AND SUBSTITUTIONS IN A SPECIFIC REGION:

The overall strategy to used to perform site-directed mutagenesis was:

Mutagenic primers (oligonucleotides) were synthesized corresponding to the DNA sequence flanking the sites of insertion and substitutions, separated by the DNA base pairs defining the insertions and substitutions.

Subsequently, the resulting mutagenic primers were used in a PCR reaction with the modified plasmid pJS3 (see above). The resulting PCR fragment was purified and extended in a second PCR-reaction, the resulting PCR product was purified and extended in a third PCR-reaction before being digested by endonucleases and cloned into the *E. coli - B. subtilis* shuttle vector (see below). The PCR reactions are performed under normal conditions.

Following this strategy two insertion and one substitution was constructed in savinase® wherein insertions was introduced in position 99 (*99aD) and 217 (*217aP) respectively and a substitution was introduced in position S99A (see below).

The insertion and substitution at position 99 was introduced by a mutagenic primer (5' CCG AAC CTG AAC CAT CCG CGG CCC CTA GGA CTT TAA CAG C 3' (*sense*)) were used in a PCR reaction with an opposite primer (5' GAG TTA AGC CCA GAA GAT GTG GAC GCG 3'(*antisense*)).

The produced PCR fragment were extended towards the C-terminal of Savinase by a second round of PCR introducing the insertion at position 217 with primer; 5' CAT CGA TGT ACC GTT TGG TAA GCT GGC ATA TGT TG 3'. The second round PCR product were extended towards the C-terminal of Savinase by a third round of PCR with primer; 5' AAC CGC ACA GCG TTT TTT TAT TGA TTA ACG CGT TGC 3', situated downstream at the Mlu I site in pJS3. All PCR reactions used plasmid pJS3 as template. The extended DNA-fragment resulting from third round PCR was cloned into the Sal I- and Mlu I- sites of the modified plasmid pJS3 (see above).

The plasmid DNA was transformed into E. coli by well-known techniques and one *E. coli* colony were sequenced to confirm the mutation designed.

In order to purify a subtilase variant of the invention, the *B. subtilis* pJS3 expression plasmid comprising a variant of the invention was transformed into a competent *B. subtilis* strain and was fermented as described above in a medium containing 10 µg/ml Chloramphenicol (CAM).

The sequence of the pJS3 plasmid is:

```
       1 AATTCCGGCC CAACGATGGC TGATTTCCGG GTTGACGGCC GGCGGAACCA AGGGGTGATC GGTCGGCGGA AATGAAGGCC TGCGGCGAGT GCGGGCCTTC
         TTAAGGCCGG GTTGCTACCG ACTAAAGGCC CAACTGCCGG CCGCCTTGGT TCCCCACTAG CCAGCCGCCT TTACTTCCGG ACGCCGCTCA CGCCCGGAAG
     101 TGTTTTGAGG ATTATAATCA GAGTATATTG AAAGTTTCGC GATCTTTTCG TATAATTGTT TTAGGCATAG TGCAATCGAT TGTTTGAGAA AAGAAGAAGA
     201 ACAAAACTCC TAATATTAGT CTCATATAAC TTTCAAAGCG CTAGAAAAGC ATATTAACAA AATCCGTATC ACGTTAGCTA ACAAACTCTT TTCTTCTTCT
     301 CCATAAAAAT ACCTTGTCTG TCATCAGACA GGGTATTTTT TATGCTGTCC AGACTGTCCG CTGTGTAAAA ATAAGGAATA AAGGGGGGTT GTTATTATTT
         GGTATTTTTA TGGAACAGAC AGTAGTCTGT CCCATAAAAA ATACGACAGG TCTGACAGGC GACACATTTT TATTCCTTAT TTCCCCCCAA CAATAATAAA
     401 TACTGATATG TAAAATATAA TTTGTATAAG AAAATGAGAG GGAGAGGAAA CATGATTCAA AAACGAAAGC GGACAGTTTC GTTCAGACTT GTGCTTATGT
         ATGACTATAC ATTTTATATT AAACATATTC TTTTACTCTC CCTCTCCTTT GTACTAAGTT TTTGCTTTCG CCTGTCAAAG CAAGTCTGAA CACGAATACA
     501 GCACGCTGTT ATTTGTCAGT TTGCCGATTA CAAAAACATC AGCCGTAAAT GGCACGCTGA TGCAGTATTT TGAATGGTAT ACGCCGAACG ACGGCCAGCA
         CGTGCGACAA TAAACAGTCA AACGGCTAAT GTTTTTGTAG TCGGCATTTA CCGTGCGACT ACGTCATAAA ACTTACCATA TGCGGCTTGC TGCCGGTCGT
     601 TTGGAAACGA TTGCAGAATG ATGCGGAACA TTTATCGGAT TAACTTAACG TTAATATTTG TTTCCCAATA GGCAAATCTT TCTAACTTTG ATACGTTTAA
         AACCTTTGCT AACGTCTTAC TACGCCTTGT AAATAGCCTA ATTGAATTGC AATTATAAAC AAAGGGTTAT CCGTTTAGAA AGATTGAAAC TATGCAAATT
     701 ACTACCAGCT TGGACAAGTT GGTATAAAAA TGAGGAGGGA AACCGAATGA AGAAACCGTT GGGGAAAATT GTCGCAAGCA CCGCACTACT CATTTCTGTT
         TGATGGTCGA ACCTGTTCAA CCATATTTTT ACTCCTCCCT TTGGCTTACT TCTTTGGCAA CCCCTTTTAA CAGCGTTCGT GGCGTGATGA GTAAAGACAA
     801 GCTTTTAGTT CATCGATCGC ATCGGCTGCT GAAGAAGCAA AAGAAAAATA TTTAATTGGC TTTAATGAGC AGGAAGCTGT CAGTGAGTTT GTAGAACAAG
         CGAAAATCAA GTAGCTAGCG TAGCCGACGA CTTCTTCGTT TTCTTTTTAT AAATTAACCG AAATTACTCG TCCTTCGACA GTCACTCAAA CATCTTGTTC
     901 TAGAGGCAAA TGACGAGGTC GCCATTCTCT CTGAGGAAGA GGAAGTCGAA ATTGAATTGC TTCATGAATT TGAAACGATT CCTGTTTTAT CCGTTGAGTT
         ATCTCCGTTT ACTGCTCCAG CGGTAAGAGA GACTCCTTCT CCTTCAGCTT TAACTTAACG AAGTACTTAA ACTTTGCTAA GGACAAAATA GGCAACTCAA
    1001 AAGCCCAGAA GATGTGGACG CGCTTGAACT CGATCCAGCG ATTTCTTATA TTGAAGAGGA TGCAGAAGTA ACGACAATGG CGCAATCGGT ACCATGGGGA
         TTCGGGTCTT CTACACCTGC GCGAACTTGA GCTAGGTCGC TAAAGAATAT AACTTCTCCT ACGTCTTCAT TGCTGTTACC GCGTTAGCCA TGGTACCCCT
    1101 ATTAGCCGTG TGCAAGCCCC AGCTGCCCAT AACCGTGGAT TGACAGGTTC TGGTGTAAAA GTTGCTGTCC TCGATACAGG GATATCCACT CATCCAGATC
         TAATCGGCAC ACGTTCGGGG TCGACGGGTA TTGGCACCTA ACTGTCCAAG ACCACATTTT CAACGACAGG AGCTATGTCC CTATAGGTGA GTAGGTCTAG
    1201 TAAATATTCG TGGTGGCGCA AGCTTTGTAC CAGGGGAACC GTCGACTCAA GATGGGAATG GGCATGGCAC GCATGTGGCC GGGACGATCG CTGCTTTAAA
         ATTTATAAGC ACCACCGCGT TCGAAACATG GTCCCCTTGG CAGCTGAGTT CTACCCTTAC CCGTACCGTG CGTACACCGG CCCTGCTAGC GACGAAATTT
    1301 CAATTCGATT GGCGTTCTTG GCGTAGCTCC TAGCGCTGAG CTATACGCTG TTAAAGTCCT AGGGGCGAGC GGTTCAGGTT CGGTCAGCTC GATTGCCCAA
         GTTAAGCTAA CCGCAAGAAC CGCATCGAGG ATCGCGACTC GATATGCGAC AATTTCAGGA TCCCCGCTCG CCAAGTCCAA GCCAGTCGAG CTAACGGGTT
    1401 GGATTGGAAT GGGCAGGGAA CAATGGCATG CACGTTGCTA ATTTGAGTTT AGGAAGCCCT TCGCCAAGTG CCACACTCGA GCAAGCTGTT AATAGCGCGA
         CCTAACCTTA CCCGTCCCTT GTTACCGTAC GTGCAACGAT TAAACTCAAA TCCTTCGGGA AGCGGTTCAC GGTGTGAGCT CGTTCGACAA TTATCGCGCT
    1501 CTTCTAGAGG CGTTCTTGTT GTAGCGGCAT CTGGGAATTC AGGTGCAGGC TCAATCAGCT ATCCGGCGCG CTATGCGAAC GCAATGGCAG TCGGAGCTAC
         GAAGATCTCC GCAAGAACAA CATCGCCGTA GACCCTTAAG TCCACGTCCG AGTTAGTCGA TAGGCCGCGC GATACGCTTG CGTTACCGTC AGCCTCGATG
    1601 TGATCAAAAC AACAACCGCG CTAGCTTTTC ACAGTATGGC GCAGGCCTTG ACATTGTCGC ACCCGGGGTA AACGTGCAGA GCACATACCC AGGTTCAACA
         ACTAGTTTTG TTGTTGGCGC GATCGAAAAG TGTCATACCG CGTCCGGAAC TGTAACAGCG TGGGCCCCAT TTGCACGTCT CGTGTATGGG TCCAAGTTGT
    1701 TATGCCAGCT TAAACGGTAC ATCGATGGCT ACTCCTCATG TTGCAGGTGC GGCCGCCCTT GTTAAACAAA AGAACCCATC TTGGTCTAAT GTACAAATTC
         ATACGGTCGA ATTTGCCATG TAGCTACCGA TGAGGAGTAC AACGTCCACG CCGGCGGGAA CAATTTGTTT TCTTGGGTAG AACCAGATTA CATGTTTAAG
    1801 GAAATCATCT AAAGAATACG GCAACTAGTT TAGGAAGCAC GAACTTGTAT GGAAGCGGAC TTGTTAACGC AGAAGCGGCA ACGCGTTAAT CAATAAAAAA
         CTTTAGTAGA TTTCTTATGC CGTTGATCAA ATCCTTCGTG CTTGAACATA CCTTCGCCTG AACAATTGCG TCTTCGCCGT TGCGCAATTA GTTATTTTTT
    1901 ACGCTGTGCG GTTAAAGGGC ACAGCGTTTT TTTGTGTATG GATCAGCTTG GCGTAATCAT GGTCATAGCT GTTTCCTGTG TGAAATTGTT ATCCGCTCAC
         TGCGACACGC CAATTTCCCG TGTCGCAAAA AAACACATAC CTAGTCGAAC CGCATTAGTA CCAGTATCGA CAAAGGACAC ACTTTAACAA TAGGCGAGTG
    2001 AATTCCACAC AACATACGAG CCGGAAGCAT AAAGTGTAAA GCCTGGGGTG CCTAATGAGT GAGCTAACTC ACATTAATTG CGTTGCGCTC ACTGCCCGCT
         TTAAGGTGTG TTGTATGCTC GGCCTTCGTA TTTCACATTT CGGACCCCAC GGATTACTCA CTCGATTGAG TGTAATTAAC GCAACGCGAG TGACGGGCGA
    2101 TTCCAGTCGG GAAACCTGTC GTGCCAGCTG CATTAATGAA TCGGCCAACG CGCGGGGAGA GGCGGTTTGC GTATTGGGCG CTCTTCCGCT TCCTCGCTCA
         AAGGTCAGCC CTTTGGACAG CACGGTCGAC GTAATTACTT AGCCGGTTGC GCGCCCTCT CCGCCAAACG CATAACCCGC GAGAAGGCGA AGGAGCGAGT
    2201 CTGACTCGCT GCGCTCGGTC GTTCGGCTGC GGCGAGCGGT ATCAGCTCAC TCAAAGGCGG TAATACGGTT ATCCACAGAA TCAGGGGATA ACGCAGGAAA
         GACTGAGCGA CGCGAGCCAG CAAGCCGACG CCGCTCGCCA TAGTCGAGTG AGTTTCCGCC ATTATGCCAA TAGGTGTCTT AGTCCCCTAT TGCGTCCTTT
    2301 GAACATGTGA GCAAAAGGCC AGCAAAAGGC CAGGAACCGT AAAAAGGCCG CGTTGCTGGC GTTTTTCCAT AGGCTCCGCC CCCCTGACGA GCATCACAAA
         CTTGTACACT CGTTTTCCGG TCGTTTTCCG GTCCTTGGCA TTTTTCCGGC GCAACGACCG CAAAAAGGTA TCCGAGGCGG GGGGACTGCT CGTAGTGTTT
    2401 AATCGACGCT CAAGTCAGAG GTGGCGAAAC CCGACAGGAC TATAAAGATA CCAGGCGTTT CCCCCTGGAA GCTCCCTCGT GCGCTCTCCT GTTCCGACCC
         TTAGCTGCGA GTTCAGTCTC CACCGCTTTG GGCTGTCCTG ATATTTCTAT GGTCCGCAAA GGGGGACCTT CGAGGGAGCA CGCGAGAGGA CAAGGCTGGG
    2501 TGCCGCTTAC CGGATACCTG TCCGCCTTTC TCCCTTCGGG AAGCGTGGCG CTTTCTCATA GCTCACGCTG TAGGTATCTC AGTTCGGTGT AGGTCGTTCG
         ACGGCGAATG GCCTATGGAC AGGCGAAAAG AGGGAAGCCC TTCGCACCGC GAAAGAGTAT CGAGTGCGAC ATCCATAGAG TCAAGCCACA TCCAGCAAGC
    2601 CTCCAAGCTG GGCTGTGTGC ACGAACCCCC CGTTCAGCCC GACCGCTGCG CCTTATCCGG TAACTATCGT CTTGAGTCCA ACCCGGTAAG ACACGACTTA
         GAGGTTCGAC CCGACACACG TGCTTGGGGG GCAAGTCGGG CTGGCGACGC GGAATAGGCC ATTGATAGCA GAACTCAGGT TGGGCCATTC TGTGCTGAAT
    2701 TCGCCACTGG CAGCAGCCAC TGGTAACAGG ATTAGCAGAG CGAGGTATGT AGGCGGTGCT ACAGAGTTCT TGAAGTGGTG GCCTAACTAC GGCTACACTA
         AGCGGTGACC GTCGTCGGTG ACCATTGTCC TAATCGTCTC GCTCCATACA TCCGCCACGA TGTCTCAAGA ACTTCACCAC CGGATTGATG CCGATGTGAT
    2801 GAAGGACAGT ATTTGGTATC TGCGCTCTGC TGAAGCCAGT TACCTTCGGA AAAAGAGTTG GTAGCTCTTG ATCCGGCAAA CAAACCACCG CTGGTAGCGG
         CTTCCTGTCA TAAACCATAG ACGCGAGACG ACTTCGGTCA ATGGAAGCCT TTTTCTCAAC CATCGAGAAC TAGGCCGTTT GTTTGGTGGC GACCATCGCC
    2901 TGGTTTTTTT GTTTGCAAGC AGCAGATTAC GCGCAGAAAA AAAGGATCTC AAGAAGATCC TTTGATCTTT TCTACGGGGT CTGACGCTCA GTGGAACGAA
         ACCAAAAAAA CAAACGTTCG TCGTCTAATG CGCGTCTTTT TTTCCTAGAG TTCTTCTAGG AAACTAGAAA AGATGCCCCA GACTGCGAGT CACCTTGCTT
    3001 AACTCACGTT AAGGGATTTT GGTCATGAGA TTATCAAAAA GGATCTTCAC CTAGATCCTT TTAAATTAAA AATGAAGTTT TAAATCAATC TAAAGTATAT
         TTGAGTGCAA TTCCCTAAAA CCAGTACTCT AATAGTTTTT CCTAGAAGTG GATCTAGGAA AATTTAATTT TTACTTCAAA ATTTAGTTAG ATTTCATATA
    3101 ATGAGTAAAC TTGGTCTGAC AGTTACCAAT GCTTAATCAG TGAGGCACCT ATCTCAGCGA TCTGTCTATT TCGTTCATCC ATAGTTGCCT GACTCCCCGT
         TACTCATTTG AACCAGACTG TCAATGGTTA CGAATTAGTC ACTCCGTGGA TAGAGTCGCT AGACAGATAA AGCAAGTAGG TATCAACGGA CTGAGGGGCA
    3201 CGTGTAGATA ACTACGATAC GGGAGGGCTT ACCATCTGGC CCCAGTGCTG CAATGATACC GCGAGACCCA CGCTCACCGG CTCCAGATTT ATCAGCAATA
         GCACATCTAT TGATGCTATG CCCTCCCGAA TGGTAGACCG GGGTCACGAC GTTACTATGG CGCTCTGGGT GCGAGTGGCC GAGGTCTAAA TAGTCGTTAT
    3301 AACCAGCCAG CCGGAAGGGC CGAGCGCAGA AGTGGTCCTG CAACTTTATC CGCCTCCATC CAGTCTATTA ATTGTTGCCG GGAAGCTAGA GTAAGTAGTT
         TTGGTCGGTC GGCCTTCCCG GCTCGCGTCT TCACCAGGAC GTTGAAATAG GCGGAGGTAG GTCAGATAAT TAACAACGGC CCTTCGATCT CATTCATCAA
    3401 CGCCAGTTAA TAGTTTGCGC AACGTTGTTG CCATTGCTAC AGGCATCGTG GTGTCACGCT CGTCGTTTGG TATGGCTTCA TTCAGCTCCG GTTCCCAACG
         GCGGTCAATT ATCAAACGCG TTGCAACAAC GGTAACGATG TCCGTAGCAC CACAGTGCGA GCAGCAAACC ATACCGAAGT AAGTCGAGGC CAAGGGTTGC
    3501 ATCAAGGCGA GTTACATGAT CCCCCATGTT GTGCAAAAAA GCGGTTAGCT CCTTCGGTCC TCCGATCGTT GTCAGAAGTA AGTTGGCCGC AGTGTTATCA
         TAGTTCCGCT CAATGTACTA GGGGGTACAA CACGTTTTTT CGCCAATCGA GGAAGCCAGG AGGCTAGCAA CAGTCTTCAT TCAACCGGCG TCACAATAGT
    3601 CTCATGGTTA TGGCAGCACT GCATAATTCT CTTACTGTCA TGCCATCCGT AAGATGCTTT TCTGTGACTG GTGAGTACTC AACCAAGTCA TTCTGAGAAT
         GAGTACCAAT ACCGTCGTGA CGTATTAAGA GAATGACAGT ACGGTAGGCA TTCTACGAAA AGACACTGAC CACTCATGAG TTGGTTCAGT AAGACTCTTA
    3701 AGTGTATGCG GCGACCGAGT TGCTCTTGCC CGGCGTCAAT ACGGGATAAT ACCGCGCCAC ATAGCAGAAC TTTAAAAGTG CTCATCATTG GAAAACGTTC
         TCACATACGC CGCTGGCTCA ACGAGAACGG GCCGCAGTTA TGCCCTATTA TGGCGCGGTG TATCGTCTTG AAATTTTCAC GAGTAGTAAC CTTTTGCAAG
    3801 TTCGGGGCGA AAACTCTCAA GGATCTTACC GCTGTTGAGA TCCAGTTCGA TGTAACCCAC TCGTGCACCC AACTGATCTT CAGCATCTTT TACTTTCACC
         AAGCCCCGCT TTTGAGAGTT CCTAGAATGG CGACAACTCT AGGTCAAGCT ACATTGGGTG AGCACGTGGG TTGACTAGAA GTCGTAGAAA ATGAAAGTGG
    3901 AGCGTTTCTG GGTGAGCAAA AACAGGAAGG CAAAATGCCG CAAAAAAGGG AATAAGGGCG ACACGGAAAT GTTGAATACT CATACTCTTC CTTTTTCAAT
         TCGCAAAGAC CCACTCGTTT TTGTCCTTCC GTTTTACGGC GTTTTTTCCC TTATTCCCGC TGTGCCTTTA CAACTTATGA GTATGAGAAG GAAAAAGTTA
    4001 GATCCTCTAC GCCGGACGCA TCGTGGCCGG CATCACCGGC GCCACAGGTG CGGTTGCTGG CGCCTATATC GCCGACATCA CCGATGGGGC AGATCGGGCT
         CTAGGAGATG CGGCCTGCGT AGCACCGGCC GTAGTGGCCG CGGTGTCCAC GCCAACGACC GCGGATATAG CGGCTGTAGT GGCTACCCCT TCTAGCCCGA
    4101 CGCCACTTCG GGCTCATGAG CGCTTGTTTC GGCGTGGGTA TGGTGGCAGG CCCGTGGCCG GGGGACTGTT GGGCGCCATC TCCTTGCATG CCTTTTAGTC
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                GCGGTGAAGC CCGAGTACTC GCGAACAAAG CCGCACCCAT ACCACCGTCC GGGCACCGGC CCCCTGACAA CCCGCGGTAG AGGAACGTAC GGAAAATCAG
        4101    CAGCTGATTT CACTTTTTGC ATTCTACAAA CTGCATAACT CATATGTAAA TCGCTCCTTT TTAGGTGGCA CAAATGTGAG GCATTTTCGC TCTTTCCGGC
                GTCGACTAAA GTGAAAAACG TAAGATGTTT GACGTATTGA GTATACATTT AGCGAGGAAA AATCCACCGT GTTTACACTC CGTAAAAGCG AGAAAGGCCG
        4201    GAGGCTAGTT ACCCTTAAGT TATTGGTATG ACTGGTTTTA AGCGCAAAAA AAGTTGCTTT TTCGTACCTA TTAATGTATC GTTAGAAAAC CGACTGTAAA
        4301    CTCCGATCAA TGGGAATTCA ATAACCATAC TGACCAAAAT TCGCGTTTTT TTCAACGAAA AAGCATGGAT AATTACATAG CAATCTTTTG GCTGACATTT
                AAGTACAGTC GGCATTATCT CATATTATAA AAGCCAGTCA TTAGGCCTAT CTGACAATTC CTGAATAGAG TTCATAAACA ATCCTGCATG ATAACCATCA
        4401    TTCATGTCAG CCGTAATAGA GTATAATATT TTCGGTCAGT AATCCGGATA GACTGTTAAG GACTTATCTC AAGTATTTGT TAGGACGTAC TATTGGTAGT
                CAAACAGAAT GATGTACCTG TAAAGATAGC GGTAAATATA TTGAATTACC TTTATTAATG AATTTTCCTG CTGTAATAAT GGGTAGAAGG TAATTACTAT
        4501    GTTTGTCTTA CTACATGGAC ATTTCTATCG CCATTTATAT AACTTAATGG AAATAATTAC TTAAAAGGAC GACATTATTA CCCATCTTCC ATTAATGATA
                TATTATTGAT ATTTAAGTTA AACCCAGTAA ATGAAGTCCA TGGAATAATA GAAAGAGAAA AAGCATTTTC AGGTATAGGT GTTTTGGGAA ACAATTTCCC
        4601    ATAATAACTA TAAATTCAAT TTGGGTCATT TACTTCAGGT ACCTTATTAT CTTTCTCTTT TTCGTAAAAG TCCATATCCA CAAAACCCTT TGTTAAAGGG
                CGAACCATTA TATTTCTCTA CATCAGAAAG GTATAAATCA TAAAACTCTT TGAAGTCATT CTTTACAGGA GTCCAAATAC CAGAGAATGT TTTAGATACA
        4701    GCTTGGTAAT ATAAAGAGAT GTAGTCTTTC CATATTTAGT ATTTTGAGAA ACTTCAGTAA GAAATGTCCT CAGGTTTATG GTCTCTTACA AAATCTATGT
                CCATCAAAAA TTGTATAAAG TGGCTCTAAC TTATCCCAAT AACCTAACTC TCCGTCGCTA TTGTAACCAG TTCTAAAAGC TGTATTTGAG TTTATCACCC
        4801    GGTAGTTTTT AACATATTTC ACCGAGATTG AATAGGGTTA TTGGATTGAG AGGCAGCGAT AACATTGGTC AAGATTTTCG ACATAAACTC AAATAGTGGG
                TTGTCACTAA GAAAATAAAT GCAGGGTAAA ATTTATATCC TTCTTGTTTT ATGTTTCGGT ATAAAACACT AATATCAATT TCTGTGGTTA TACTAAAAGT
        4901    AACAGTGATT CTTTTATTTA CGTCCCATTT TAAATATAGG AAGAACAAAA TACAAAGCCA TATTTTGTGA TTATAGTTAA AGACACCAAT ATGATTTTCA
                CGTTTGTTGG TTCAAATAAT GATTAAATAT CTCTTTTCTC TTCCAATTGT CTAAATCAAT TTTATTAAAG TTCATTTGAT ATGCCTCCTA AATTTTTATC
        5001    GCAAACAACC AAGTTTATTA CTAATTTATA GAGAAAAGAG AAGGTTAACA GATTTAGTTA AAATAATTTC AAGTAAACTA TACGGAGGAT TTAAAAATAG
                TAAAGTGAAT TTAGGAGGCT TACTTGTCTG CTTTCTTCAT TAGAATCAAT CCTTTTTTAA AAGTCAATAT TACTGTAACA TAAATATATA TTTTAAAAAT
        5101    ATTTCACTTA AATCCTCCGA ATGAACAGAC GAAAGAAGTA ATCTTAGTTA GGAAAAAATT TTCAGTTATA ATGACATTGT ATTTATATAT AAAATTTTTA
                ATCCCACTTT ATCCAATATT CGTTCCTTAA TTTCATGAAC AATCTTCATT CTTTCTTCTC TAGTCATTAT TATTGGTCCC AGATCTGGTT GAACTACTCT
        5201    TAGGGTGAAA TAGGTTATAA GCAAGGAATT AAAGTACTTG TTAGAAGTAA GAAAGAAGAG ATCAGTAATA ATAACCAGGG TCTAGACCAA CTTGATGAGA
                TTAATAAAAT AATTTTTCCG TTCCCAATTC CACATTGCAA TAATAGAAAA TCCATCTTCA TCGGCTTTTT CGTCATCATC TGTATGAATC AAATCGCCTT
        5301    AATTATTTTA TTAAAAAGGC AAGGGTTAAG GTGTAACGTT ATTATCTTTT AGGTAGAAGT AGCCGAAAAA GCAGTAGTAG ACATACTTAG TTTAGCGGAA
                CTTCTGTGTC ATCAAGGTTT AATTTTTTAT GTATTTCTTT TAACAAACCA CCATAGGAGA TTAACCTTTT ACGGTGTAAA CCTTCCTCCA AATCAGACAA
        5401    GAAGACACAG TAGTTCCAAA TTAAAAAATA CATAAAGAAA ATTGTTTGGT GGTATCCTCT AATTGGAAAA TGCCACATTT GGAAGGAGGT TTAGTCTGTT
                ACGTTTCAAA TTCTTTTCTT CATCATCGGT CATAAAATCC GTATCCTTTA CAGGATATTT TGCAGTTTCG TCAATTGCCG ATTGTATATC CGATTTATAT
        5501    TGCAAAGTTT AAGAAAAGAA GTAGTAGCCA GTATTTTAGG CATAGGAAAT GTCCTATAAA ACGTCAAAGC AGTTAACGGC TAACATATAG GCTAAATATA
                TTATTTTTCG GTCGAATCAT TTGAACTTTT ACATTTGGAT CATAGTCTAA TTTCATTGCC TTTTTCCAAA ATTGAATCCA TTGTTTTTGA TTCACGTAGT
        5601    AATAAAAAGC CAGCTTAGTA AACTTGAAAA TGTAAACCTA GTATCAGATT AAAGTAACGG AAAAAGGTTT TAACTTAGGT AACAAAAACT AAGTGCATCA
                TTTCTGTATT CTTAAAATAA GTTGGTTCCA CACATACCAA TACATGCATG TGCTGATTAT AAGAATTATC TTTATTATTT ATTGTCACTT CCGTTGCACG
        5701    AAAGACATAA GAATTTTATT CAACCAAGGT GTGTATGGTT ATGTACGTAC ACGACTAATA TTCTTAATAG AAATAATAAA TAACAGTGAA GGCAACGTGC
                CATAAAACCA ACAAGATTTT TATTAATTTT TTTATATTGC ATCATTCGGC GAAATCCTTG AGCCATATCT GACAAACTCT TATTTAATTC TTCGCCATCA
        5801    GTATTTTGGT TGTTCTAAAA ATAATTAAAA AAATATAACG TAGTAAGCCG CTTTAGGAAC TCGGTATAGA CTGTTTGAGA ATAAATTAAG AAGCGGTAGT
                TAAACATTTT TAACTGTTAA TGTGAGAAAC AACCAACGAA CTGTTGGCTT TTGTTTAATA ACTTCAGCAA CAACCTTTTG TGACTGAATG CCATGTTTCA
        5901    ATTTGTAAAA ATTGACAATT ACACTCTTTG TTGGTTGCTT GACAACCGAA AACAAATTAT TGAAGTCGTT GTTGGAAAAC ACTGACTTAC GGTACAAAGT
                TTGCTCTCCT CCAGTTGCAC ATTGGACAAA GCCTGGATTT ACAAAACCAC ACTCGATACA ACTTTCTTTC GCCTGTTTCA CGATTTTGTT TATACTCTAA
        6001    AACGAGAGGA GGTCAACGTG TAACCTGTTT CGGACCTAAA TGTTTTGGTG TGAGCTATGT TGAAAGAAAG CGGACAAAGT GCTAAAACAA ATATGAGATT
                TATTTCAGCA CAATCTTTTA CTCTTTCAGC CTTTTTAAAT TCAAGAATAT GCAGAAGTTC AAAGTAATCA ACATTAGCGA TTTTCTTTTC TCTCCATGGT
        6101    ATAAAGTCGT GTTAGAAAAT GAGAAAGTCG GAAAAATTTA AGTTCTTATA CGTCTTCAAG TTTCATTAGT TGTAATCGCT AAAAGAAAAG AGAGGTACCA
                CTCACTTTTC CACTTTTTGT CTTGTCCACT AAAACCCTTG ATTTTTCATC TGAATAAATG CTACTATTAG GACACATAAT ATTAAAAGAA ACCCCCATCT
        6201    GAGTGAAAAG GTGAAAAACA GAACAGGTGA TTTTGGGAAC TAAAAAGTAG ACTTATTTAC GATGATAATC CTGTGTATTA TAATTTTCTT TGGGGGTAGA
                ATTTAGTTAT TTGTTTAGTC ACTTATAACT TTAACAGATG GGGTTTTTCT GTGCAACCAA TTTTAAGGGT TTTCAATACT TTAAAACACA TACATACCAA
        6301    TAAATCAATA AACAAATCAG TGAATATTGA AATTGTCTAC CCCAAAAAGA CACGTTGGTT AAAATTCCCA AAAGTTATGA AATTTTGTGT ATGTATGGTT
                CACTTCAACG CACCTTTCAG CAACTAAAAT AAAAATGACG TTATTTCTAT ATGTATCAAG ATAAGAAAGA ACAAGTTCAA AACCATCAAA AAAAGACACC
        6401    GTGAAGTTGC GTGGAAAGTC GTTGATTTTA TTTTTACTGC AATAAAGATA TACATAGTTC TATTCTTTCT TGTTCAAGTT TTGGTAGTTT TTTTCTGTGG
                TTTTCAGGTG CTTTTTTTAT TTTATAAACT CATTGGGTGA TCTCGACTTC GTTCTTTTTT TACCTCTCGG TTATGAGTTA GTTCAAATTC GTTCTTTTTA
        6501    AAAAGTCCAC GAAAAAAATA AAATATTTGA GTAACCCACT AGAGCTGAAG CAAGAAAAAA ATGGAGAGCC AATACTCAAT CAAGTTTAAG CAAGAAAAAT
                GGTTCTAAAT CGTGTTTTTC TTGGAATTGT GCTGTTTTAT CCTTTACCTT GTCTACAAAC CCCTTAAAAA CGTTTTTAAA GGCTTTTAAG CCGTCTGTAC
                CCAAGATTTA GCACAAAAAG AACCTTAACA CGACAAAATA GGAAATGGAA CAGATGTTTG GGGAATTTTT GCAAAAATTT CCGAAAATTC GGCAGACATG
        6601    GTTCCTTAAG G
                CAAGGAATTC C
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EXAMPLE 2

PURIFICATION OF ENZYME VARIANTS:

This procedure relates to purification of a 2 liter scale fermentation for the production of the subtilases of the invention in a *Bacillus* host cell.

Approximately 1.6 liters of fermentation broth were centrifuged at 5000 rpm for 35 minutes in 1 liter beakers. The supernatants were adjusted to pH 6.5 using 10% acetic acid and filtered on Seitz Supra S100 filter plates.

The filtrates were concentrated to approximately 400 ml using an Amicon CH2A UF unit equipped with an Amicon S1Y10 UF cartridge. The UF concentrate was centrifuged and filtered prior to absorption at room temperature on a Bacitracin af-

finity column at pH 7. The protease was eluted from the Bacitracin column at room temperature using 25% 2-propanol and 1 M sodium chloride in a buffer solution with 0.01 dimethylglutaric acid, 0.1 M boric acid and 0.002 M calcium chloride adjusted to pH 7.

The fractions with protease activity from the Bacitracin purification step were combined and applied to a 750 ml Sephadex G25 column (5 cm dia.) equilibrated with a buffer containing 0.01 dimethylglutaric acid, 0.2 M boric acid and 0.002 m calcium chloride adjusted to pH 6.5.

Fractions with proteolytic activity from the Sephadex G25 column were combined and applied to a 150 ml CM Sepharose CL 6B cation exchange column (5 cm dia.) equilibrated with a buffer containing 0.01 M dimethylglutaric acid, 0.2 M boric acid, and 0.002 M calcium chloride adjusted to pH 6.5.

The protease was eluted using a linear gradient of 0-0.1 M sodium chloride in 2 litres of the same buffer (0-0.2 M sodium chloride in case of Subtilisin 147).

In a final purification step protease containing fractions from the CM Sepharose column were combined and concentrated in an Amicon ultrafiltration cell equipped with a GR81PP membrane (from the Danish Sugar Factories Inc.).

By using the techniques of Example 1 for the construction and fermentation, and the above isolation procedure the following subtilisin 309 variants were produced and isolated:

L217LP + S99SD + S99A

EXAMPLE 3

The "MODEL DETERGENT WASH PERFORMANCE TEST":

In order to asses the wash performance of selected subtilase variants in a standard detergent composition, standard washing experiments may be performed using the below experimental conditions:

Detergent:	Model detergent
Detergent dosage	4.0 g/l
pH	10.1
Wash time	20 min
Temperature:	30°C
Water hardness:	15°dH
Enzyme concentration:	10 nm (in the detergent solution)
Test system:	10 ml beakers with a stirring rod
Textile/volume:	5 textile pieces (Ø 2.5 cm)/50 ml detergent solution
Test material:	WFK10N (egg stains)

The composition of the model detergent is as follows:

6.2%	LAS (Nansa 80S)
2%	Sodium salt of C_{16}-C_{18} fatty acid
4%	Non-ionic surfactant (Plurafax LF404)
22%	Zeolite P
10.5%	Na_2CO_3
4%	$Na_2Si_2O_5$
2%	Carboxymethylcellulose (CMC)
6.8%	Acrylate liquid CP5 40%
20%	Sodium perborate (empirical formula $NaBO_2.H_2O_2$)
0.2%	EDTA
21%	Na_2SO_4
	Water (balance)

pH of the detergent solution is adjusted to 10.1 by addition of HCl or NaOH. Water hardness is adjusted to 15°dH by addition of $CaCl_2$ and $MGCl_2$ (Ca^{2+}:Mg^{2+} = 4:1) to the test system. After washing the textile pieces are flushed in tap water and air-dried.

Measurement of the reflectance ($R_{variant}$) on the test material is performed at 460 nm using a Macbeth ColorEye 7000 photometer (Macbeth, Division of Kollmorgen Instruments Corporation, Germany). The measurements are performed accordance with the manufacturer's protocol.

In order to determine a blank value, a similar wash experiment is performed without addition of enzyme. The subsequent measurement of the reflectance (R_{blank}) is performed as described right above.

A reference experiment is then performed as described above, wherein the wash performance of the parent enzyme is tested. The subsequent measurement of the reflectance (R_{parent}) is performed as described right above.

The wash performance is evaluated by means of the Performance Factor (P) which is defined in accordance with the below formula:

$$P = (R_{variant} - R_{blank}) - (R_{parent} - R_{blank})$$
$$= R_{variant} - R_{parent}.$$

EXAMPLE 4

THE "OVO-INHIBITION ASSAY"

The below inhibition assay is based on the principle that the subtilase variant to be tested will catalyse the hydrolysis of a peptide-pNA bond, thereby releasing the yellow pNA, which may

conveniently be followed at 405 nm. The amount of released pNA after a given period of time is a direct measure of the subtilase activity. By carrying out such hydrolysis experiments with and without inhibitor, respectively, it is possible to obtain a quantitative measure for the degree to which a certain subtilase variant is inhibited.

Reaction conditions:

Enzyme concentration:	0.0003 mg/ml
Conc. of trypsin inhibitor type IV-0:	0.0015 mg/ml
Initial substrate concentration:	0.81 mM
Reaction time:	11 min
Assay temperature:	25°C
Assay pH:	8.6
Absorbance measured at:	405 nm

Assay solutions:

Substrate solution (2 mM): 500 mg Suc-Ala-Ala-Pro-Phe-pNA is dissolved in 4 ml DMSO (200 mM). This solution is diluted 100 times with the buffer solution described below. The concentration of substrate in the resulting substrate solution is 2 mM.

Inhibitor solution (0.005 mg/ml): 5 mg trypsin inhibitor type IV-0 (Sigma T-1886) is dissolved in 10 ml water. This solution is dissolved 100 times with the buffer solution described below. The concentration of inhibitor in the resulting inhibitor solution is 0.005 mg/ml.

Enzyme solution (0.001 mg/ml): 1 mg enzyme is dissolved in 10 ml water. This solution is dissolved 100 times with the buffer solution described below. The concentration of enzyme in the resulting enzyme solution is 0.001 mg/ml.

Buffer solution (pH 8.6): 15.7 mg Tris is dissolved in an appropriate amount of water and 0.75 ml 30% (w/v) BRIJ (BRIJ 35 polyoxyethylenelaurylether, 30% (w/v), Sigma Cat. No. 430AG-6) is added. The pH is adjusted to 8.6 with 4 M NaOH and the solution is diluted to 1 liter with water.

Assay with inhibitor

1 volume unit (e.g. 80 µl) inhibitor solution is mixed with 1 volume unit (e.g. 80 µl) enzyme solution in an appropriate reaction vessel (e.g. a spectrophotometer cell or a micro titer plate) and equilibrated at 25°C for 15 min. 1.375 volume units (e.g. 110 µl) substrate solution is added to the reaction vessel after which the absorbance at 405 nm is followed for 11 min (e.g. by measuring every 10^{th} or 30^{th} second). The slope of the absorbance curve is calculated using linear regression analysis. The slope of the absorbance curve is denoted $\alpha_{inhibitor}$.

Assay without inhibitor

1 volume unit (e.g. 80 µl) buffer solution is mixed with 1 volume unit (e.g. 80 µl) enzyme solution in an appropriate reaction vessel (e.g. a spectrophotometer cell or a micro titer plate) and equilibrated at 25°C for 15 min. 1.375 volume units (e.g. 110 µl) substrate solution is added to the reaction vessel after which the absorbance at 405 nm is followed for 11 min (e.g. by measuring every 10^{th} or 30^{th} second). The slope of the absorbance curve is calculated using linear regression analysis. The slope of the absorbance curve is denoted α.

Blank

1 volume unit (e.g. 80 µl) inhibitor solution is mixed with 1 volume unit (e.g. 80 µl) buffer solution in an appropriate reaction vessel (e.g. a spectrophotometer cell or a micro titer plate) and equilibrated at 25°C for 15 min. 1.375 volume units

(e.g. 110 µl) substrate solution is added to the reaction vessel after which the absorbance at 405 nm is followed for 15 min. These measurements are not used in the calculations, but merely serve as a control that no enzyme has been added to the buffer and/or substrate solution.

Calculation of Residual Activity (RA)

The residual enzyme activity (RA) is calculated according to the below formula:

$$RA = (\alpha_{inhibitor}/\alpha) \times 100\%$$

Using the above test, the following results were obtained:

Enzyme	Residual Activity (%)
Savinase®	<5%
L217LP + S99SD + S99A	97.0%

CLAIMS

1. A subtilase variant selected from the group consisting of

a subtilase variant comprising an insertion of at least one additional amino acid residue between positions 42 and 43 (BASBPN numbering);

a subtilase variant comprising an insertion of at least one additional amino acid residue between positions 51 and 55 (BASBPN numbering);

a subtilase variant comprising an insertion of at least one additional amino acid residue between positions 155 and 160 (BASBPN numbering);

a subtilase variant comprising an insertion of at least one additional amino acid residue between positions 187 and 189 (BASBPN numbering);

a subtilase variant comprising an insertion of at least one additional amino acid residue between positions 216 and 217 (BASBPN numbering); and

a subtilase variant comprising an insertion of at least one additional amino acid residue between positions 217 and 218 (BASBPN numbering); and

a subtilase variant comprising an insertion of at least one additional amino acid residue between positions 218 and 219 (BASBPN numbering).

2. The variant according to claim 1, wherein the insertion is selected from the group consisting of: insertion between posi-

tions 51 and 52, insertion between positions 52 and 53, insertion between positions 53 and 54, insertion between positions 54 and 55, and insertion between positions 55 and 56 (BASBPN numbering).

3. The variant according to claim 1, wherein the insertion is selected from the group consisting of: insertion between positions 155 and 156, insertion between positions 156 and 157, insertion between positions 157 and 158, insertion between positions 158 and 159, insertion between positions 159 and 160, and insertion between positions 160 and 161 (BASBPN numbering).

4. The variant according to claim 1, wherein the insertion is selected from the group consisting of: insertion between positions 187 and 188, insertion between positions 188 and 189, and insertion between positions 189 and 190 (BASBPN numbering).

5. The variant according to any of the preceding claims, where the variant - when tested in the "Ovo-inhibition Assay" disclosed in Example 4 herein - has a Residual Activity of at least 10%.

6. The variant according to claim 5, where the variant has a Residual Activity of at least 15%, preferably at least 20%, more preferably at least 25%.

7. The variant according to any of the preceding claims, wherein the variant contains one insertion.

8. The variant according to any of claims 1-6, wherein the variant contains two insertions.

9. The variant according to any of claims 1-6, wherein the variant contains more than two insertions.

10. The variant according to any of the preceding claims, wherein the variant further comprises at least one modification.

11. The variant according to claim 10, wherein the modification is a substitution.

12. The variant according to claim 1, wherein the insertion is selected from the group consisting of X42XA, X42XT, X42XG, X42XS, X42XD, X42XE, X42XK, X42XR, X42XH, X42XV, X42XC, X42XN, X42XQ, X42XF, X42XI, X42XL, X42XM, X42XP, X42XW and X42XY.

13. The variant according to claim 2, wherein the insertion is selected from the group consisting of X51XA, X51XT, X51XG, X51XS, X51XD, X51XE, X51XK, X51XR, X51XH, X51XV, X51XC, X51XN, X51XQ, X51XF, X51XI, X51XL, X51XM, X51XP, X51XW and X51XY.

14. The variant according to claim 2, wherein the insertion is selected from the group consisting of X52XA, X52XT, X52XG, X52XS, X52XD, X52XE, X52XK, X52XR, X52XH, X52XV, X52XC, X52XN, X52XQ, X52XF, X52XI, X52XL, X52XM, X52XP, X52XW and X52XY.

15. The variant according to claim 2, wherein the insertion is selected from the group consisting of X53XA, X53XT, X53XG, X53XS, X53XD, X53XE, X53XK, X53XR, X53XH, X53XV, X53XC, X53XN, X53XQ, X53XF, X53XI, X53XL, X53XM, X53XP, X53XW and X53XY.

16. The variant according to claim 2, wherein the insertion is selected from the group consisting of X54XA, X54XT, X54XG, X54XS, X54XD, X54XE, X54XK, X54XR, X54XH, X54XV, X54XC, X54XN, X54XQ, X54XF, X54XI, X54XL, X54XM, X54XP, X54XW and X54XY.

17. The variant according to claim 2, wherein the insertion is selected from the group consisting of X55XA, X55XT, X55XG, X55XS, X55XD, X55XE, X55XK, X55XR, X55XH, X55XV, X55XC, X55XN, X55XQ, X55XF, X55XI, X55XL, X55XM, X55XP, X55XW and X55XY.

18. The variant according to claim 3, wherein the insertion is selected from the group consisting of X155XA, X155XT, X155XG, X155XS, X155XD, X155XE, X155XK, X155XR, X155XH, X155XV, X155XC, X155XN, X155XQ, X155XF, X155XI, X155XL, X155XM, X155XP, X155XW and X155XY.

19. The variant according to claim 3, wherein the insertion is selected from the group consisting of X156XA, X156XT, X156XG, X156XS, X156XD, X156XE, X156XK, X156XR, X156XH, X156XV, X156XC, X156XN, X156XQ, X156XF, X156XI, X156XL, X156XM, X156XP, X156XW and X156XY.

20. The variant according to claim 3, wherein the insertion is selected from the group consisting of X157XA, X157XT, X157XG, X157XS, X157XD, X157XE, X157XK, X157XR, X157XH, X157XV, X157XC, X157XN, X157XQ, X157XF, X157XI, X157XL, X157XM, X157XP, X157XW and X157XY.

21. The variant according to claim 3, wherein the insertion is selected from the group consisting of X158XA, X158XT, X158XG, X158XS, X158XD, X158XE, X158XK, X158XR, X158XH, X158XV, X158XC, X158XN, X158XQ, X158XF, X158XI, X158XL, X158XM, X158XP, X158XW and X158XY.

22. The variant according to claim 3, wherein the insertion is selected from the group consisting of X159XA, X159XT, X159XG, X159XS, X159XD, X159XE, X159XK, X159XR, X159XH, X159XV, X159XC,

X159XN, X159XQ, X159XF, X159XI, X159XL, X159XM, X159XP, X159XW and X159XY.

23. The variant according to claim 3, wherein the insertion is selected from the group consisting of X160XA, X160XT, X160XG, X160XS, X160XD, X160XE, X160XK, X160XR, X160XH, X160XV, X160XC, X160XN, X160XQ, X160XF, X160XI, X160XL, X160XM, X160XP, X160XW and X160XY.

24. The variant according to claim 4, wherein the insertion is selected from the group consisting of X187XA, X187XT, X187XG, X187XS, X187XD, X187XE, X187XK, X187XR, X187XH, X187XV, X187XC, X187XN, X187XQ, X187XF, X187XI, X187XL, X187XM, X187XP, X187XW and X187XY.

25. The variant according to claim 4, wherein the insertion is selected from the group consisting of X188XA, X188XT, X188XG, X188XS, X188XD, X188XE, X188XK, X188XR, X188XH, X188XV, X188XC, X188XN, X188XQ, X188XF, X188XI, X188XL, X188XM, X188XP, X188XW and X188XY.

26. The variant according to claim 4, wherein the insertion is selected from the group consisting of X189XA, X189XT, X189XG, X189XS, X189XD, X189XE, X189XK, X189XR, X189XH, X189XV, X189XC, X189XN, X189XQ, X189XF, X189XI, X189XL, X189XM, X189XP, X189XW and X189XY.

27. The variant according to claim 1, wherein the insertion is selected from the group consisting of X216XA, X216XT, X216XG, X216XS, X216XD, X216XE, X216XK, X216XR, X216XH, X216XV, X216XC, X216XN, X216XQ, X216XF, X216XI, X216XL, X216XM, X216XP, X216XW and X216XY.

28. The variant according to claim 1, wherein the insertion is selected from the group consisting of X217XA, X217XT, X217XG, X217XS, X217XD, X217XE, X217XK, X217XR, X217XH, X217XV, X217XC, X217XN, X217XQ, X217XF, X217XI, X217XL, X217XM, X217XP, X217XW and X217XY.

29. The variant according to claim 1, wherein the insertion is selected from the group consisting of X218XA, X218XT, X218XG, X218XS, X218XD, X218XE, X218XK, X218XR, X218XH, X218XV, X218XC, X218XN, X218XQ, X218XF, X218XI, X218XL, X218XM, X218XP, X218XW and X218XY.

30. The variant according to any of the preceding claims, wherein the parent subtilase belongs to the sub-group I-S1.

31. The variant according to claim 30, wherein the parent subtilase is selected from the group consisting of BSS168, BSSDY and BLSCAR, or functional variants thereof having retained the characteristics of sub-group I-S1.

32. The variant according to any of claims 1-29, wherein the parent subtilase belongs to the sub-group I-S2.

33. The variant according to claim 32, wherein the parent subtilase is selected from the group consisting of BLS147, BLSAVI (savinase®), BAPB92, TVTHER and BYSYAB, or functional variants thereof having retained the characteristics of sub-group I-S2.

34. The variant according to claim 33, wherein the parent subtilase is BLSAVI (savinase®).

35. The variant according to any of claims 32-34, wherein the variant further comprises at least one modification in the positions 27, 36, 56, 76, 87, 95, 96, 97, 98, 99, 100, 101, 102,

103, 104, 120, 123, 159, 167, 170, 206, 218, 222, 224, 232, 235, 236, 245, 248, 252 or 274 (BASBPN numbering).

36. The variant according to claim 35, wherein the variant further comprises the modification S101G+S103A+V104I+G159D+A232V+Q236H+Q245R+N248D+N252K.

37. The variant according to any of claims 32-36, wherein the variant further comprises at least one additional amino acid residue in the active site loop (b) region from position 95 to 103 (BASBPN numbering).

38. An isolated DNA sequence encoding a subtilase variant as defined in any of claims 1-37.

39. An expression vector comprising the isolated DNA sequence of claim 38.

40. A microbial host cell transformed with the expression vector of claim 39.

41. A microbial host cell according to claim 40, which is a bacterium, preferably a Bacillus, especially a *B. lentus*.

42. A microbial host cell according to claim 40, which is a fungus or yeast, preferably a filamentous fungus, especially an Aspergillus.

43. A method for producing a subtilase variant as defined in any of claims 1-37, wherein a host as defined in any of claims 40-42 is cultured under conditions conducive to the expression and secretion of the variant, and the variant is recovered.

44. A cleaning or detergent composition, preferably a laundry or dishwash composition, comprising the variant as defined in any of claims 1-37.

45. A composition according to claim 44, which additionally comprises a cellulase, a lipase, a cutinase, an oxidoreductase, another protease, an amylase or a mixture thereof.

46. Use of a variant as defined in any of claims 1-37 in a laundry and/or a dishwash detergent.

47. A method for removal of egg stains from a hard surface or from laundry, the method comprising contacting the egg stain-containing hard surface or the egg stain-containing laundry with a cleaning or detergent composition, preferably a laundry or dishwash composition, which contains a subtilase variant as defined in any of claims 1-37.

48. A method according to claim 47, wherein the composition additionally comprises a cellulase, a lipase, a cutinase, an oxidoreductase, another protease, an amylase or a mixture thereof.

49. Use of a cleaning or detergent composition, preferably a laundry or dishwash composition, containing a subtilase variant as defined in any of claims 1-37 for removal of egg stains from laundry or from hard surfaces.

50. Use according to claim 49, wherein the composition additionally comprises a cellulase, a lipase, a cutinase, an oxidoreductase, another protease, an amylase or a mixture thereof.

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No: 1         10         20         30         40         50
a)  AQSVPYGVSQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLKVAGGASM
b)  AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGI*STHPDLNIRGGASF

No:          60         70         80         90         100
a)  VPSETNPFQDNNSHGTHVAGTVAALNNSIGVLGVAPSASLYAVKVLGADG
b)  VPGEPST*QDGNGHGTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGASG

No:          110        120        130        140        150
a)  SGQYSWIINGIEWAIANNMDVINMSLGGPSGSAALKAAVDKAVASGVVVV
b)  SGSVSSIAQGLEWAGNNGMHVANLSLGSPSPSATLEQAVNSATSRGVLVV

No:          160        170        180        190        200
a)  AAAGNEGTSGSSSTVGYPGKYPSVIAVGAVDSSNQRASFSSVGPELDVMA
b)  AASGNSG*AGS***ISYPARYANAMAVGATDQNNNRASFSQYGAGLDIVA

No:          210        220        230        240        250
a)  PGVSIQSTLPGNKYGAYNGTSMASPHVAGAAALILSKHPNWTNTQVRSSL
b)  PGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHL

No:          260        270  275
a)  ENTTTKLGDSFYYGKGLINVQAAAQ
b)  KNTATSLGSTNLYGSGLVNAEAATR
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Fig.1